Metabolomics and cell-based assay analysis of selected South African plants to determine anti-Rift Valley Fever Virus activity

by

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Table of contents

Chapter 2

Epidemiology, pathogenesis, transmission cycles and reactive oxygen species in Rift Valley Fever virus

Chapter 3

Cytotoxicity, antiviral and radical scavenging activity of selected antiviral medicinal plants

Chapter 4

^{1}H-NMR-based metabolomics and UHPLC-qTOF-MS analysis of plants with anti-RVFV activity

List of figures

List of tables

List of abbreviations

Reagents and media	
RVFV	Rift Valley Fever Virus
DMEM	Dulbecco's modified Eagles' medium
FBS	Fetal bovine serum
Pen/strep	Penicillin/streptomycin
MTT	3-[4,5-dimethyl-2-thiazol-yl]-2,5-diphenyl-2H-tetrazolium bromide
LPS	Lipopolysaccharide
CO_2	Carbon dioxide
H2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
DPPH	2,2-diphenyl-1-picrylhydrazyl
$ABTS^+$	2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ROS	Reactive oxygen species
IC_{50}	Inhibitory concentration 50 %
DMSO	Dimethyl sulfoxide
MeOH	Methanol
TMS	Tetramethyl silane
TSP	Trimethyl silane propionic acid sodium salt
Units of measure	
°C	Degree celsius
mg	Milligram
L	Litre
LC_{50}	Lethal concentration 50%
µg	Microgram
ppm	Parts per million
Statistics	
SD	Standard Deviation
PCA	Principal Component Analysis
OPLS-DA	Orthogonal Projections to Latent Structures – Discriminant Analysis

HCA	Hierarchical Cluster Analysis
Instruments	
^{1}H-NMR	Proton - Nuclear Magnetic Resonance
UHPLC	Ultra-High-Performance Liquid Chromatography
qTOF-MS	Quadrupole Time-of-Flight Mass Spectrometry

Declaration of independent work

I Garland Kgosi More hereby declare that this research project submitted to the University of South Africa (UNISA), Florida, Johannesburg for the Degree of Doctor of Philosophy in Agriculture, is my own independent work. It complies with the research ethics and the code of academic integrity. It has not been previously submitted by me for a degree at this or any other institution.

Signature …… Date ……

Memoriam

In memory of

Mrs. Catherine Dina Moswetsa

My biggest cheerleader, prayer warrior, my grandmother.

You always looked forward to my success.

Your prayers have sustained me.

Professor Jacques Vervoort

Prof. Vervoort was always a good spirited person, passionate with a lively personality. He was instrumental in steering this study towards contributing to the body of science through his analytical methods expertise.

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Preface

Structure of the thesis

Chapter 1. This introductory chapter discusses the ethnopharmacological aspects of plants as source of therapeutics in traditional practice in Africa and worldwide. Furthermore, it highlights the pharmacological effects and phytochemistry of selected medicinal plants scientifically screened for antiviral activity. Emerging analytical technologies used for metabolomics with a perspective on metabolites identification from plants is presented.

Chapter 2. A thorough discussion of the epidemiology, pathogenesis and transmission of the Rift Valley Fever Virus (RVFV) is offered in this chapter. Moreover, this chapter seeks to convey the role of reactive oxygen species in RVFV infection and current treatments.

Chapter 3. This chapter presents the screening of 20 medicinal plants selected for their pharmacologically and biological activity against RVFV and to assess their toxicity on Vero cells. The chapter also covers the cell-based antioxidant assays with an attempt to reduce the reactive oxygen species in LPS stimulated cells.

Chapter 4. Here the application of ^{1}H-NMR metabolomics combined with multivariate statistical data analysis for profiling of metabolites in eight most active antiviral medicinal plant species is described. Data obtained from the UHPLC-qTOF-MS was correlated using databases (KEGG, PubChem, HMDB, NIST, DNP, MassBank, mzCloud, and MAGMa) and metabolites contributing to the activity were putatively identified.

Chapter 5. The discussion and conclusion chapter summarise the work presented in this thesis into drug discovery perspectives, recommendations, and future prospects.

Chapter 6. The appendix provides additional dose response graphs and ^{1}H-NMR spectra of tested samples.

Peer reviewed publications

More, G.K.; Makola, R.T.; Prinsloo, G. In-vitro Evaluation of Anti-Rift Valley Fever Virus, Antioxidant and Anti-Inflammatory Activity of South African Medicinal Plant Extracts. *Viruses* **2021**, *13*, 221. https://doi.org/10.3390/v13020221

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Garland Kgosi More, Jacques Vervoort, Paul Anton Steenkamp, Gerhard Prinsloo, Metabolomic Profile of Medicinal Plants with Anti-RVFV Activity

Abstract

Rift Valley Fever Virus (RVFV) is a mosquito-borne haemorrhagic fever virus affecting both humans and animals with severe morbidity and mortality. RVFV is an emerging virus that is endemic to Africa. However, it can infect a variety of mosquito species and therefore spread to other parts of the world. Despite the efforts made to reduce or curb the RVFV infection and other tropical diseases in recent years, the re-occurrence of the RVFV may devastate the world economically, bearing in mind the shortage of therapeutic agents. There is an urgent need for novel anti-RVFV chemotherapeutic agents that can circumvent the acquired diseases. Emergence of new viruses such as the currently tormenting Coronavirus (SARS-CoV-2), which are challenging to control due to the mutative nature of the viral genome, highlights the importance for this type of research.

The current study investigated the anti-RVFV, cytotoxicity, and radical scavenging activities of the 50 % aqueous-methanolic leaf extracts of twenty plant species pharmacologically proven to exhibit antiviral activities. The cytotoxicity assay was performed to determine the concentration range of extracts for the subsequent study of antiviral activity to be tested in the non-toxic range in Vero cells. A green monkey kidney (Vero) cell line was used to investigate the cytotoxicity of the extracts using the 3-[4,5-dimethyl-2-thiazol-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell viability method and all plant extracts tested exhibited lethal concentrations (LC_{50}) values > 20 µg/mL which signifies that the extracts were relatively non-toxic. Determination of the anti-RVFV activity of the plant extracts was performed using the 50% tissue culture infectious dose ($TCID_{50}$) assay of tenfold serial dilutions of the virus and extracts in Vero cells. The extracts were screened in quadruplicate in 96-well tissue culture plates. After seven days, the cytopathic effect (CPE) was analysed using a light microscope and the $TCID_{50}$ was calculated. This study demonstrated promising anti-RVFV activity of eight extracts (*Artemisia afra*, *Adansonia digitata*, *Euclea natalensis*, *Elaeodendron croceum*, *Elaeodendron transvaalensis*, *Elephantorrhiza elephantina*, *Helichrysum aureonitens*, *Sutherlandia frutescens*) which exerted reduction of the viral load with $TCID_{50} < 10^5$. Radical scavenging activity of extracts was executed on 96 well plates

utilizing the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) scavenging assays. Furthermore, cell-based radical scavenging potential was done. The cell-permeant probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and Griess reagent assays were used to evaluate the effects of extracts on LPS-induced ROS and RNS production, respectively. The H_2DCF-DA fluorescence intensity indicative of ROS production in images acquired using the Laser Scanning Confocal Microscope was observed. The EC_{50} values were calculated with non-linear regression analysis with a variable slope using GraphPad Prism software version 8.0 (GraphPad Software La Jolla, CA, USA). The results showed that the extracts quenched free radicals exhibiting an EC_{50} value range of 4.12–20.41 µg/mL and suppressed the level of pro-inflammatory mediators by 60–80 % in Vero cells. These plant extracts were shown to exhibit nontoxicity to Vero cells, but showed antiviral potency against RVFV. Moreover, extracts reduced the level of ROS which might contribute in preventing disease propagation via oxidative stress reduction.

^{1}H-NMR-based metabolomics on samples from twenty medicinal plant species was conducted with consistent data processing and multivariate statistical analysis using MestReNova and SIMCA software, respectively. The principal component analysis (PCA), the orthogonal projections to latent structures- discriminant analysis (OPLS-DA) and hierarchical clustering analysis (HCA) plots were constructed to assess the distribution and discrimination of the samples. The variables on the far ends of the S-plot and variable importance in projection (VIP) score values > 1, were considered significant as potential biomarkers. Thorough monitoring of the performance of the model was performed using the explained variation (R^2), predicted variation (Q^2) coefficients, cross-validated prediction residuals (CV-ANOVA), *p* values < 0.05, 100 x permutation and receiver operating characteristic (Area Under the Curve) [ROC(AUC)] > 7 %. ^{1}H-NMR spectral peaks were interpreted and annotated with the Human Metabolome Database (HMDB) and Chenomx software.

Furthermore, extracts from eight plant species with potent antiviral activity were subjected to the UHPLC-qTOF-MS analysis to investigate possible metabolites responsible for the activity. The UHPLC-qTOF-MS instrument was operated in dual (positive and negative) electrospray ionisation (ESI) mode. Spectral data such as mass spectra, retention time (Rt) and ion fragments from the UPLC-qTOF-MS analysis was searched against several databases including Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.jp/kegg/),

PubChem (https://pubchemblog.ncbi.nlm.nih.gov/), Human Metabolome Database (HMDB: http://www.hmdb.ca/), NIST (National Institute of Standards and Technology) database, DNP (Dictionary of Natural Products: www.dnp.chemnetbase.com), MassBank (USA), mzCloud (Advanced Mass Spectral Database), and MAGMa (www.emetabolomics.org) for putative identification of metabolites. In addition, findings from the analysis were further compared to peer reviewed literature. A total of 61 putative metabolites in the samples were detected. However, of most importance is the annotation of two fatty acids, 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid and 13-hydroxy-9Z,11E-octadecadienoic acid detected in the negative ionisation mode which are present in all active samples. Our results suggest that these annotated metabolites of interest may be responsible for the antiviral activity observed. This study provided a scientific evaluation of the efficacy of medicinal plants against RVFV, provided possible avenues for mechanisms of action and demonstrated potential of using ^{1}H-NMR-metabolomics in combination with UHPLC-qTOF-MS for rapid drug discovery.

Chapter 1

Ethnopharmacology, pharmacology and phytochemistry of selected antiviral medicinal plants

1.1 A brief overview of ethnopharmacology and pharmacology

Ethnopharmacology is the study of ancient traditional use of plants as natural therapies for the treatment and management of infectious diseases. This was practiced extensively in developing countries to produce remedies for the treatment of diseases instigated by either bacteria, fungus, viruses and parasites. These medicinal plants were used in the following forms: crude, ground powders, teas, tinctures, poultices (Balick and Cox, 1997), and they were mainly used by traditional healers in areas such as Asia, India and Africa (Van Vuuren & Viljoen, 2011, Van Wyk, 2011). Flora has played a crucial role in primary health care as they are an affordable way to treat various diseases. About 70–95 % of the world's population in developing countries' rely greatly on plants as treatment for their primary health care diseases (World Health Organisation, 2019). The practice of using plants as medicine has, however, captured the interest of many researchers for isolating phytochemicals that are relevant to the pharmaceutical industry.

The history of the isolation and identification of phytochemicals began with the isolation of morphine, atropine, codeine, cocaine, digitoxin and quinine, to mention a few (Newman et al., 2000; Butler, 2004). This has attracted an interest in the investigations of the biological activities in the plants used in traditional medicines to find therapeutic leads for the treatment of ailments such as and not limited diabetes, cancer, tuberculosis, HIV/AIDS, diarrhoea, malaria, cholera, eczema and Alzheimer's (Süntar, 2019). Throughout history, the advent of Western medicines marginalized many of the traditional medicinal plant uses (Fitzgerald et al., 2019). This led to a polarized market with synthetic formulated medicine prescribed by a professional pharmacist and synthesised pharmaceuticals gaining the attention of the financially privileged. Meanwhile, the disadvantaged could only afford tinctures, teas, poultices and powders prepared by traditional healers (Van Wyk & Gorelik, 2017).

Despite advances in synthetic organic chemistry, plant-derived medicinal products have also been a leading source in drug development, that over 50 % of the best-selling drugs used in modern times are derivatives of natural products. The highly prescribed plant-based medicines are either sold as formulations of extracts or formulations of pure isolated compounds as the main ingredient. Some examples of extract formulations may include plants such as *Harpogophytum procumbens* (Devil's claws), *Ginkgo biloba* (Ginkgo), *Catharanthus roseus* (Periwinkle), *Prunus africana* (African plum), *Sutherlandia frutescens* and *Lobostemon*

fruticosus and many products from *Aloe* species. Furthermore, there are approximately 100 plant-derived products approved for medicinal purposes (Table 1.1) (Pan et al., 2013).

Recently there has been a growing need for the use of indigenous medicinal plants worldwide, as such, the development of ethnopharmacology is expected to bring economic benefits and may protect cultural heritage. In paving the way for economic benefits, conservation of the cultural heritage and mutual benefit sharing of traditional knowledge in South Africa, a collaboration between institutions of research and traditional healers has been established (Bagley, 2018). This initiative aims to exploit known medicinal plants, as well as screening unique southern African plants, more generally in search of pharmacologically active compounds. Therefore, the latter partnership has created more effective exploitation of medicinal plants (Reihling, 2008). Moreover, there are over 200 000 registered traditional healers recognised as the primary informants of indigenous knowledge and participants in drug discovery (Galvan et al., 2016). This serves to prevent the exploitation of traditional healers by a research institution and curbs the over-harvesting of medicinal plants which potentiates extinction. Also, stringent regulations should be put in place to safeguard and govern the harvesting and trading of plants, especially the highly threatened species (Rasethe, Semenya & Maroyi, 2019).

Table 1.1 Plant-derived pharmaceuticals and their applications (Pan et al., 2013).

Plant	Drug	Uses
Galanthus woronowii	Galanthamine	Alzheimer's disease
Podophyllum peltatum	Podophyllotoxin	Anticancer
Artemisia annua	Artemisinin	Antimalaria
Taxus brevifolia	Taxol	Anticancer
Cannabis sativa	Cannabidiol, Delta-9-tetrahydrocannabinol	Antiemetic, pain management, appetite stimulant
Silybum marianum	Silymarim	Hepatic disorder
Tricosanthes kirilowii	Tricisanthin	Anticancer, antiviral

1.2 Medicinal plants as a source of antiviral therapeutics

The advent of communicable diseases and resistant strains have placed immense challenges to public health in different countries especially developing countries and an urge to develop new therapeutics is inevitable. Viruses including HIV, HSV, hepatitis, influenza and arthropod-borne viruses such as dengue virus, cytomegalovirus, RVFV and recently coronaviruses SARS-CoV-2 (CSGICTV, 2020), are challenging to control due to mutative nature of the viral genomes. Therefore, due to the high prevalence of viral infections for which there are no adequate treatments and due to constant appearance of new resistant viral strains, the development of novel antiviral agents is essential. Many traditional medicinal plants including those in Table 1.2 have been reported to have antiviral activity (Kapoor et al., 2017; Akram et al., 2018). Several of these medicinal plants and their constituents have complementary and overlapping mechanisms of action (Babar et al., 2013).These include:

- Immunomodulation, which involves the stimulation of immune response to release natural killer cells (NK) and cytokines to fight against the viral pathogen (Rojas et al., 2017; Flórez-álvarez et al., 2018).
- Viral entry inhibitors and viral attachments. The capability of the virus to cleave and enter the host cell is an important process that is currently the target for many types of research focusing on developing effective therapeutic drugs. In HIV infection,

disrupting the interaction of the virus with the Chemokine receptors 5 (CCR-5) receptors prevent the attachment and therefore incapacitate the viral entry to the host cell (Razonable et al., 2011).

- Replication and translation inhibition; these inhibitors prevent enzymes like reverse transcriptase and integrase from replicating or transcribing the viral DNA into the host DNA (Malys & McCarthy, 2011). This process has been the major focus of many drugs discovery research worldwide.
- Viral assembly and release inhibitors; these types of inhibitor prevent protease enzyme from processing of proteins which is important in cell-to-cell transmission (Lee et al., 2012).

The aim of this study was to evaluate the antiviral activity using 50 % aqueous-methanolic extracts of 20 different plant species (Table 1.2) known to possess antiviral properties against RVFV. These plants were selected based on literature especially ethnopharmacological uses and *in-vitro* pharmacological antiviral activities. The chemical composition of each plant was investigated to determine antiviral compounds from the active extracts.

Table 1.2 Selected pharmacologically active antiviral plants, their family names, and antiviral activities.

Plant names	Family	Antiviral activities	References
Adansonia digitata	Malvaceae	HSV-1 NDV HSV-1; ASFV HIV1 RT, HIV-FRET, PR	Rathore et al, 2007 Sulaiman et al., 2011 Silva et al., 1997 Sharma & Rangari, 2016
Artemisia afra	Asteraceae	HIV-1/2	Asres et al., 2001; Liu et al., 2009
Aloe ferox	Asphodelaceae	HSV-1	Ferox & Somnifera, 2007
Carissa edulis	Apocynaceae	HSV-1 CDV, CPIV, FHV, LSDV PV-2 CMV	Tolo et al., 2006; Mukhtar et al., 2008 Bagla et al., 2012 Robin et al., 2002 Tolo et al., 2007
Crinum macowanii	Amaryllidaceae	HIV-1 RT, PR	Klos et al., 2009
Elaeodendron croceum	Celastraceae	HIV-CB	Prinsloo et al., 2010
Elaeodendron transvaalense	Celastraceae	HIV-1 a-Glucosidase, RT, CB, NF-kB, Tat, IN	Bessong et al., 2006, 2005; Tshikalange et al., 2008
Elephantorrhiza elephantina	Fabaceae	HIV-RT	Sigidi et al., 2017
Euclea natalensis	Ebenaceae	HIV-1 RT HSV-1	Lall et al., 2005
Helichrysum aureonitens	Asteraceae	HSV-1, Cox B-1, Ad31 reovirus	Meyer et al., 1997
Hyteropyxis natalensis	Heteropyxidaceae	HIV-1 RT	Hurinanthan et al., 2013
Lobostemon fruticosus	Boraginaceae	HIV-1	Lunat, 2011

Moringa oleifera	Moringaceae	HSV1 HIV-1 RT FMDV HBV EBV	Lipipun et al., 2003; Hafidh et al., 2009 Ali et al., 2002 Younus et al., 2015 Feustel et al., 2017 Tshabalala et al., 2019
Peltophorum africanum	Fabaceae	HIV1-RT	Bessong et al., 2005; Theo et al., 2009
Prunus africana	Rosaceae	CMV	Tolo et al., 2007
Ricinus communis	Euphorbiaceae	HIV1- RT, RNase H, IN	Wang and Ng, 2001; Bessong et al., 2005, 2006; El-Toumy et al., 2018
Senna petersiana	Fabaceae	HIV1-RT	Tshikalange et al., 2008
Sutherlandia frutescens	Fabaceae	HIV1 RT, IN, RNase H	Harnett et al., 2005; Bessong et al., 2005, 2006; Van Wyk & Albrecht, 2008
Terminalia sericea	Combretaceae	HIV1 RT HIV-1 RNA-dependent-DNA polymerase (RDDP)	Tshikalange et al., 2008 Bessong et al., 2006
Ziziphus mucronata	Rhamnaceae	HIV-1 RT, RNase H	Bessong et al., 2005

Human immune deficiency virus (HIV-1,2; RT- Reverse transcriptase, PR- protease, CB- cell-based assay, FRET- fluorescence resonance energy transfer), Herpes simplex virus type (HSV-1,2), Feline herpesvirus-1 (FHV-1), Newcastle disease virus (NDV), Hepatitis B Virus (HBV), Canine distemper virus (CDV), Canine parainfluenza virus-2 (CPIV-2), Poliovirus (PV-2), Cytomegalovirus (CMV), Lumpy skin disease virus (LSDV), Coxsackie B virus (COX B-1), Adenovirus 31 (AD-31), African swine fever virus (ASFV), Foot and Mouth disease virus (FMDV), Epstein-Barr virus (EBV).

1.3 Phytochemistry of selected medicinal plants

Plants have naturally evolved over more than hundred million of the years and developed a survival mechanism that includes morphological characteristics and chemical diversity to fight against insects, animal, and microbial attack, this has made the plant kingdom a rich diversity of secondary metabolites with a vast pharmacological spectrum. Some major phytochemical classes include terpenoids, flavonoids, lignans, sulphides, coumarins, phenolics. saponins, alkaloids, proteins and peptides to mention a few, are reported to have been found in the plants and they possess antiviral activities (Mukhtar et al., 2008b; Babar et al., 2013; Ruwali et al., 2013; Zaynab et al., 2018; Prinsloo & Vervoort, 2018).

1.3.1 *Adansonia digitata* L

Baobab is the largest succulent tree in Africa which belongs to the family Malvaceae. It is characterized by a greyish-brown layer of stem bark and hand-sized leaves that are rich in vitamin C. Large fruits are covered in yellowish-brown hairs and it contains a white powdery substance and kidney-shaped seeds. *Adansonia digitata* is a multipurpose medicinal plant, traditionally used for treating various ailments such as fever, diarrhoea, malaria, kidney infection, bladder diseases, smallpox, measles and haemoptysis (Van Wyk & Gericke, 2000). The methanol stem bark extract has shown inhibitory effects of HIV-RT with IC_{50} of 23.6 µg/mL (Sharma & Rangari, 2016) and the stem bark extracted with methanol displayed inhibitory activity against *Plasmodium berghei*-infected mice. The extract showed chemo-suppressive effects at 400 mg kg^{-1} b.wt. (Adeoye & Bewaji, 2015). Tembo et al., (2017) has reported the presence of metabolites such as epicatechin **(1)**, gallic acid **(2),** procyanidin **(3)** and hydroxycinnamic acid glycosides from the fruit (Li et al., 2017).

1.3.2 *Aloe ferox* Mill.

Aloe ferox is a tall single-stemmed plant consisting of spanned margins of the leaves and flowers which vary in colours and have spike-like heads. This plant is widely found over the southwestern Cape, southern KwaZulu-Natal into the Free State and southern Lesotho (Van Wyk, Van Oudtshoorn, and Gericke, 1997; Chen et al., 2012). It has been registered in the red-list of South African endangered plants due to its over-harvesting that leads to extinction (Mogale, Raimondo & Van Wyk, 2019). *Aloe ferox* is well known for its many medicinal

applications such as laxative properties, skin disorders, wound healing, arthritis, sinusitis as well as conjunctivitis, ophthalmia, digestive problems, shingles and herpes (Van Wyk and Gericke, 2000). Evidence-based laboratory experiments have shown that *A. ferox* can inhibit loperamide-induced constipation in Wistar rats (Wintola et al., 2010). Aloin-A, aloe-emodin and chrysophanol were extracted from the leaves of *A. ferox* (Kambizi et al., 2007). Chen et al., (2012) described the different classes of compounds from *A. ferox* which includes aloesin **(4)**, aloeresin-C **(5)**, aloeresin-A, 5-hydroxyaloin, aloin-A **(6)**, aloin-B, aloinoside-B, and aloinoside-A by HPLC chromatography and other metabolites like quinic acid, and malic acid elucidated by NMR spectrometry.

1.3.3 *Artemisia afra* Jacq. ex Willd.

Lengana (SeTswana) is an aromatic shrub with a distinct strong sweet smell that consists of a dark-green top surface and a pale-green lower surface of the leaves. It has yellow flowers and erect stems. The plant is well known in South Africa for the treatment of coughs, colds, fever, headaches, earache, toothache, haemorrhoids, wounds, boils and malaria (Mabogo, 1990; Rabe and Van Staden, 1997; Van Wyk et al., 1997;). Anti-microbial activity in this plant have been extensively reported (Rabe and Van Staden, 1997; More et al., 2012) to exhibit anti-oxidant activity (Sunmonu et al., 2012), anti-histaminic and narcotic analgesic (Hutchings et al., 1996), hypotensive and anti-tuberculosis effects (WHO, 2020). Van Wyk et al., (2000) reported the presence of compounds in the volatile oils such as α-thujone, 1,8-cineole, β-thujone, camphor **(7)** and borneol **(8)**, scopoletin **(9)**, betulinic acid **(10)**, acacetin, phytol, α-amyrin and 12α,4α-dihydroxybishopsolicepolide were extracted from the aerial parts of *A. afra* (More et al., 2012).

1.3.4 *Carissa edulis* Vahl

This plant is a thorny shrub with shiny leaves, white flowers and edible egg-shaped fruits. It is widely spread in different African regions within the continent. Furthermore, it is widely distributed in provinces such as Mpumalanga and Limpopo provinces in South Africa (Van Wyk & Van Wyk, 2007). It's root infusion is used for chest complaints, stomach aches, rheumatism, edema, gonorrhea, syphilis, rabies and treatment of coughs (Al-youssef & Hassan, 2014). Carissin compound found in the roots of *C. edulis* has activity against cancer and Herpes simplex virus. Investigations of the root bark extract showed protection against convulsions at

20 mg/kg (Ya'U et al., 2015). Antiviral activity of oils produced from this plant tested against HSV type 1 in Vero cell culture has showed the potential of decreasing the plaque-forming units of HSV-1 (pfu) (Gavanji, et al., 2015). *Carissa edulis* has shown inhibitory effects on HSV *in-vitro* and *in vivo* (Tolo et al., 2006). Phytochemicals isolated from the ethyl-acetate fraction of this plant include quercetin-3-*O*-*β*-d-glucopyranoside, kaempferol 3-*O*-*β*-d-glucopyranoside, isorhamnetin-3-*O*-*β*-d-glucopyranoside, 3-*O*-acetyl chlorogenic acid, and rhamnetin-3-*O*-*β*-d-glucopyranoside. The butanol fractions, 1-{1-[2-(2 hydroxypropoxy) propoxy] propan-2-yloxy} propan-2-ol, isorhamnetin-3-*O*-*β*-d-glucopyranoside-(2″→1‴)-rhamnopyranoside and (+) butyl-*O*-α-l-rhamnoside were obtained (Al-Youssef & Hassan, 2017).

1.3.5 *Crinum macowanii* Baker

Commonly known as Intelezi, *C. macowanii* is a bulbous perennial plant with large undulated leaves and scented white-pink flowers. It is distributed over east, central and southern Africa. The bulb is used to treat ailments such as inflammation, respiratory system problems, kidney infections, bladder infections, venereal diseases, diarrhoea, acne, itchy rashes, rheumatic fever, boils, sores, backache and tuberculosis (Nair & Van Staden, 2013; Van Wyk and Gericke, 2007; Hutchings 1996). Because of its wide spectrum of therapeutic uses, a decrease in numbers of plants has been reported and this is due to the overharvesting of the plant (Mogale et al., 2019). Pharmacological studies ranging from antibacterial, antifungal, anti-plasmodial and cardiovascular effects have been conducted and the plant and have shown remarkable results (Mugabo et al., 2014; Mugwagwa et al., 2015). The methanol extract of the bulb was proven to decrease the cytopathic effects induced by the virus in yellow fever virus infected Vero cells by 100 % (Duri, Scovill & Huggins, 1994). The phytochemical investigations conducted have revealed that alkaloids are the major class of metabolites found in *C. macowanii* responsible for most bioactivities (Mugabo et al., 2014) and these include lycorine **(11)**, galanthamine **(12)** and crinine alkaloids.

1.3.6 *Elaeodendron croceum* (Thunb.) DC.

This is an evergreen tree with green leathery leaves and greyish stem bark. In South Africa it is distributed in the Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga and Western Cape provinces of South Africa (Archer and Van Wyk, 1998). Ethnobotanically, the

plant has been used for dyeing and tanning of clothes and animal skin for decoration (Watt and Breyer-Brandwijk 1962). (Odeyemi & Afolayan, 2017) evaluated the biological activities of *E. croceum* using an agar well diffusion method against bacterial pathogens including *S. typhimurium S. flexneri*, *S. aureus*, and *E. faecalis* and found that the tested strains were susceptible to the leaf and stem extract. They further associated the presence of flavonoids, proanthocyanidins, alkaloids, saponins and phenolics with the observed activities. A cardiac glycoside that showed activity on HIV-RT was isolated from the ethanolic extract known as digitoxigenin-glucoside (Prinsloo et al., 2010). The bark and root have been reported to be poisonous to humans (Cock & Vuuren, 2020) and cytotoxic metabolites such as 20-hydroxy-20-epi-tingenone, tingenone **(13)**, tingenine B **(14)**, 11-alpha-hydroxy-beta-amyrin **(15)** and naringenin **(16)** evaluated on Vero cells were isolated from *E. croceum* (Yelani et al., 2010).

1.3.7 *Elaeodendron transvaalense* (Burtt Davy) R.H.Archer

Elaeodendron transvaalense is a shrub that is broadly proliferated in temperate forests in eastern region of South Africa (Van Wyk, 2000). It has a smooth greyish stem bark and clustered oblong leaves with flowers that consist of three petals and three stamens (Van Wyk, and Van Wyk, 1997; Archer & Van Wyk, 1998). In ethnopharmacology, the plant is used for the treatment and management of ailments including stomach related complaints and fever, herpes simplex/zoster virus, stroke, rash and haemorrhoids (Van Wyk and Gericke, 2000). Biological screening of *E. transvaalensis* extract using HIV-RT assay showed remarkable inhibitory capacity and this led to the isolation of four triterpenes namely, methyl-epigallocatechin **(17)**, atraric acid **(18),** atranorin **(19)** and β-sitosterol (Tshikalange et al., 2005). Other metabolites such as cassinine, elaeocyanidin and canophyllol isolated from this plant have been documented (Maroyi, 2019).

1.3.8 *Elephantorrhiza elephantina* (Burch.) Skeels

The hallmark of the *E. elephantina* name lays within its ‘elephant roots’ referring to its large underground stem. This perennial shrub is characterised by its alternate, bipinnately compounded leaves and bisexual flowers (Schmidt et al., 2002). *Elephantorrhiza elephantina* is mostly found in grasslands of Botswana, Swaziland, Mozambique, Namibia, Lesotho Zimbabwe, and South Africa (Maroyi, 2017a). In South Africa, it is used traditionally for management of acne (Van Wyk and Van Wyk, 2013; Mpofu et al., 2014), blood pressure, chest

pains, stomach complains including diarrhoea (Mpofu et al., 2014) and for the treatment of fever (Kose et al., 2015). Many pharmacologically important metabolites which are anthraquinones, kaempferol **(20)**, catechin **(21)**, gallic acid **(2)** and quercetin **(22)** have been isolated from various parts of this plant (Mthembu, 2007; Mpofu et al., 2014),

1.3.9 *Euclea natalensis* A.DC.

Euclea natalensis has been widely used traditionally to treat pleurisy, asthma, bronchitis, urinary tract infections, headache, chest ailments, and toothache (Hutchings et al., 1996; Van Wyk, 2008). It's distribution is limited to the arid and rocky areas of southern Africa (Notten, 2010; Maroyi, 2017b). *In-vitro* screening reported antifungal (Van Vuuren, 2010), antibacterial, antimycobacterial (Lall and Meyer, 2001; Van der Kooy et al., 2006), antiviral (Lall et al., 2005), anti-diabetic (Deutschlnder et al., 2009), larvicidal and anti-plasmodial (Clarkson et al., 2004) activity. Several phytochemicals responsible for these activities have been isolated and identified and these include classes such as pentacyclic terpenoids (Omara et al., 2020) and the compound naphthoquinone **(23)** (Van der Kooy et al., 2006).

1.3.10 *Helichrysum aureonitens* Sch.Bip.

Helichrysum aureonitens is a perennial plant that is characterised by hairy silver-green leaves, gold flowers and slender erect stems. It is widely distributed over South Africa experiencing summer rainfall in Limpopo, Gauteng, Free State, Mpumalanga, North-West, Eastern Cape provinces and KwaZulu-Natal and Lesotho (Fabian and Germishuizen, 1997). In the indigenous knowledge systems, *H. aureonitens* is renowned for its use as an incense (Pooley, 1998) and to treat enuresis in children, skin infections, menstrual pain and wounds (Hutchings & Van Staden 1994; Hutchings et al., 1996). Secondary metabolites isolated from this plant include triyne, chlorophenol (Ziaratnia et al., 2009) and galangin **(24)** (Meyer et al., 1997).

1.3.11 *Heteropyxis natalensis* Harvey

The lavender tree is a semi-deciduous tree that has dense dark green elliptic leaves and is characterised by a pungent aromatic smell. It has flowers that form dry yellowish capsules and grey bark which turns flaky cream-white when the plant matures. The fruits are

tiny and spherical and split to release seeds (Van Wyk et al., 2002). It is a traditional medicine used to treat colds, menorrhagia, epistaxis, and gingival haemorrhage (Watt and Breyer-Brandwijk., 1962; Hutching., 1996). *In vitro, H. natalensis* has shown antibacterial activity against oral organisms (Henley-Smith et al., 2018). Essential oil from this plant contains mainly β-ocimene, 1.8-cineole, limonene, borneol, linalool, α-phellandrene, α-terpiniol and β-myrcene (Gundidza et al., 1993). Adesanwo et al, (2009) isolated a chalcone from the dichloromethane leaf extract of *H. natalensis.*

1.3.12 *Lobostemon fruticosus* (L.) H. Buek

Known as pajama bush (English), *L. fruticosus* is an evergreen shrub consisting of bluish-pink bell-shaped flowers. The alternate leaves have a rough surface and woody hairy stems. The plant is abundant in sandy soils of Namaqualand and Eastern Cape (Smith, 2003). It is used to alleviate ringworm and wound healing. For the treatment STI's, *L. fruticosus* is used in combination with *Melianthus major* and *Galenia africana* (Van Wyk et al., 1997). Motadi and Ndlovu, (2015) evaluated the anticancer activity of *L. fruticosus* and this study lead to the isolation and characterisation of two anticancer compounds which are camptothecin **(25)** and taxol **(26)**.

1.3.13 *Moringa oleifera* Lam.

Moringa is known for its broad spectrum of medicinal properties and is renowned a multipurpose plant rich in minerals such as vitamins (A, B, C, D), potassium, zinc, calcium, magnesium, iron, copper and folic acid, pyridoxine, nicotinic acid and iron (Kasolo et al., 2010; Mbikay, 2012; Oyeyinka and Oyeyinka, 2018). Laboratory tests have shown different parts of *M. oleifera* exhibit biological activities such as antiviral, anti-bacterial, anti-cancer, anti-diabetic, antioxidant, and hepatoprotective properties (Divi et al., 2012; Nair and Van Staden, 2013). Phytoconstituents like alkaloids, flavonoids, saponins, glycosides, anthraquinones, sterols, terpenoids, and tannins found in various parts of the plant (Berkovich et al., 2013). Other phytochemicals isolated from *Moringa* include niaziminin, 4-(α-L-rhamnopyranosyloxy)-benzyl isothiocyanate, methyl *N*-4-(α-L-rhamnopyranosyloxy)-benzyl carbamate, and 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl thiocarboxamide and isolated from the endophytic fungus of *Moringa* is griseofulvin **(27)**, dechlorogriseofulvin **(28)**, 8-dihydroramulosin **(29)** and mullein **(30)** (Zhao et al., 2012).

1.3.14 *Peltophorum africanum* Sond.

Native to southern Africa, *P. africanum* is a deciduous tree which grows mainly in temperate tropical regions. It consists of oblong bipinnate arranged leaves and bisexual yellow flowers (Van Wyk and Gericke, 2000). It is well known in traditional medical systems as a plant that can treat pains, venereal diseases, dysentery, toothache, diarrhoea, skin blisters, infertility, anthelmintic infections and depression (Mabogo, 1990; Ishmael, 2013; Mazimba, 2014). Pharmacological screening of this plant includes antibacterial, antifungal (Okeleye et al., 2013), anthelmintic (Bizimenyera et al., 2008), α-glucosidase inhibition (Shai et al., 2011) and anti-HIV-RT (Bessong et al., 2005), activities to mention a few. Theo et al., (2009) isolated betulinic acid from *P. africanum* which exhibited remarkable anti-HIV activity. Other isolated compounds include umbelliferone **(31)** (Mazimba, 2014); berginin **(32)** (Bam, 1988), rutin **(33)** (Ebada et al., 2008), catechin **(21)** (Bessong et al., 2005) and mangiferin **(34)** (Ebada et al., 2008).

1.3.15 *Prunus africana* (Hook. f.) Kalkman

Prunus africana, commonly known in isizulu as nyazangoma-elimnyam has pale green shiny leaves which are alternately arranged with serrated margins and consist of a grooved brown bark (Rasethe et al., 2019). Flowers are white, and the purplish fruits are round. It is found in the tropical forest of South Africa, Mozambique and Tanzania. Due to over harvesting, *P. africana* is registered in the Red List of South African plants for its over-exploitation for medicinal purposes (Stewart, 2003). *Prunus africana* is used by native people of South Africa to treat chest pains, infections (Van Wyk et al., 1997) and benign prostatic hyperplasia (Gathumbi et al., 2002). *Prunus africana* has shown to possess inhibitory activities on benign prostatic hyperplasia *in-vitro* and has shown to lower the cholesterol in the blood (Komakech et al., 2017). Major constituents including ferulic acid esters phytosterols, and pentacyclic triterpenoids have shown various biological activities (Kadu et al., 2012; Nyamai et al., 2015).

1.3.16 *Ricinus communis* L.

Ricinus communis is perennial flowering shrub with soft woody stems. It is regarded as an invasive plant in South Africa and other tropical regions. Mokhura (sePedi) is characterized by shiny, dark green or reddish leaves with palm-shaped and serrated margins.

Flowers are bright red with soft spines. It is well known for its pharmaceutically important oil from the seed known as castor oil (Severino et al., 2012). *In-vitro* antimicrobial of the plant has been documented (Naz and Bano, 2012), acaricidal (Ghosh et al., 2013), anti-diabetic (Gad-Elkareem et al., 2019) and antioxidant activity (Kadri et al., 2011). Several secondary metabolites isolated from this plant are epicatechin, ricinine **(35)**, linolenic acid **(36)** and phlobatannins (Ribeiro et al., 2016). The whole plant is considered poisonous because of the presence of ricin **(37)** (Lopez et al., 2017).

1.3.17 *Senna petersiana* (Bolle) Lock

Senna petersiana (Munembenembe, Venda name) is a deciduous shrub which consists of a brown, rough stem and alternately arranged leaves. Leaves are elliptic and hairy on the lower surface; flowers are yellow, and pods are curvy brown. It is widespread in the northern parts of Mpumalanga, Limpopo and KwaZulu-Natal. It is used ethnopharmacologically as laxative and purgative to alleviate intestinal worms, also to treat sexually transmitted diseases and constipation (Van Wyk et al., 2009). Antimicrobial and antifungal activities have been reported (Laher et al., 2013), including anti-schistosomiasis (Sparg et al., 2000), malaria (Silva et al., 2011) and sexually transmitted microbes. This study resulted in the isolation of an active ingredient, luteolin (Tshikalange et al., 2005).

1.3.18 *Sutherlandia frutescens* (L.) R.Br.

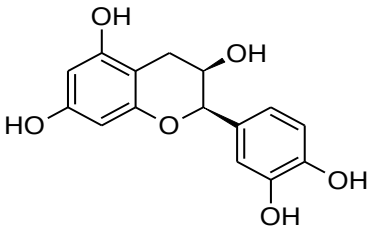
Commonly known as Cancer bush, *S. frutescens* is well known for its wide-spread use for cancer treatment in ethnopharmacology. Historically, folks used different part of the tree for the treatment and management of fever, asthma, dysentery, gastritis, diabetes, and modern research has shown it immune boosting abilities to help fight diseases (Van Wyk and Albrecht, 2008). Pharmacologically, the plant has shown remarkable biological activities including anti-cancer (Tai et al., 2004; Na et al., 2004), anti-HIV (Harnett et al., 2005) and anti-diabetes (Ojewole, 2007). Canavanine **(38)** showed reverse transcriptase (RT) inhibition and pinitol **(39)** is responsible for the anti-diabetic (Bates et al., 2000) and inflammatory activity (Kundu et al., 2005; Ojewole, 2007).

1.3.19 *Terminalia sericea* Burch. ex DC

Terminalia sericea is a small deciduous tree that is characterized by its silvery leaves, corrugated brown stem and winged seeds (Van Wyk and Gericke, 2007). Ethnopharmacologically *T. sericea* has been used to treat menstrual pains, infertility, venereal diseases, menstrual cycle problems (Bruschi et al., 2011), measles, bilharzia, diarrhoea, and gonorrhoea, (Semenya et al., 2013). The reported pharmacological activities varies from antimicrobial (Moshi and Mbwambo, 2005; Mongalo et al., 2015), antifungal (Samie and Mashau, 2013), antioxidant and anti-diabetic (Tshikalange et al., 2008; Nkobole et al., 2011) to mention a few. Saponins (Steenkamp et al., 2004), phenolic acids and amino acids (Chivandi et al., 2013) were isolated from different parts of the plant.

1.3.20 *Ziziphus mucronata* Willd.

Ziziphus mucronata is a multipurpose tree widely distributed in the sub-Saharan Africa in temperate and tropical climates. The green leaves are alternately arranged with serrated margins and the stem is mostly crenelated. Twigs have thorns at the nodes, and they bear edible oval-shaped reddish fruits (Hutchings et al., 1994). It is to treat ailments such as pains, gonorrhoea, diarrhoea, rheumatism, respiratory infections, snake bites, as an expectorant in cough, chest problems, and dysentery (Amusan et al., 2007). Its antimicrobial, antioxidant (Olajuyigbe and Afolayan, 2012), antifungal (Runyoro et al., 2006) anti-diabetic (Deutschlander et al., 2009; Mousinho et al., 2013) and acetylcholinesterase inhibitor activity have been reported (Adewusi and Steenkamp, 2011). Some phytochemical constituents responsible for the pharmacological activities observed include phenols and flavonoids (Janbaz et al., 2002). Cyclopeptide alkaloids such as mucronine-D, mucronine-j **(40)** and its derivatives were isolated from *Z. mucronata* roots (Barboni et al., 1994).



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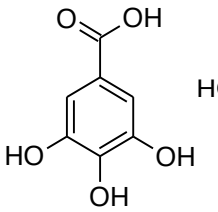

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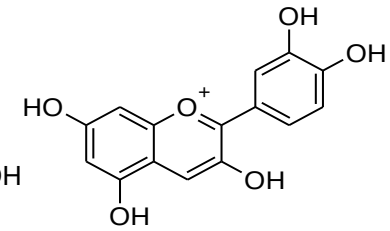

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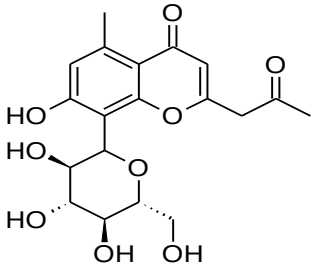

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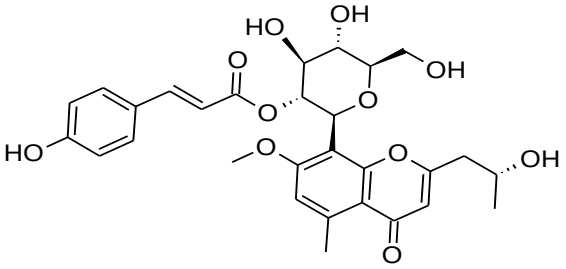

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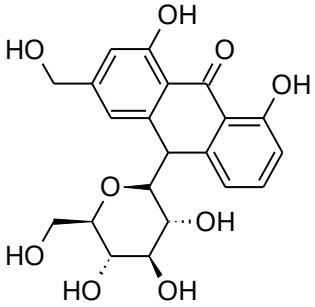

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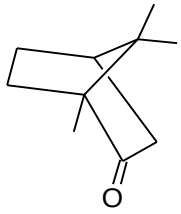

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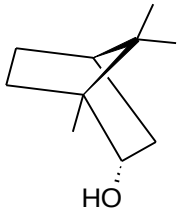

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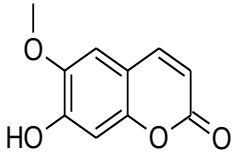

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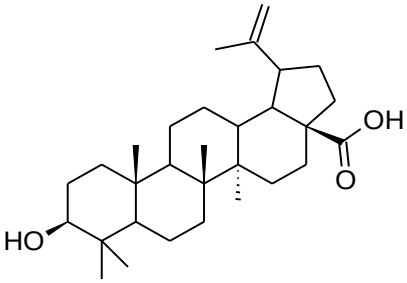

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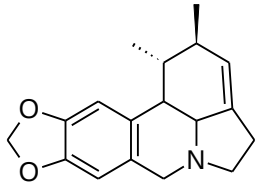

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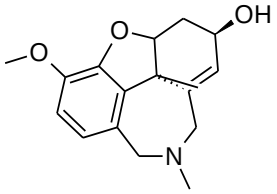

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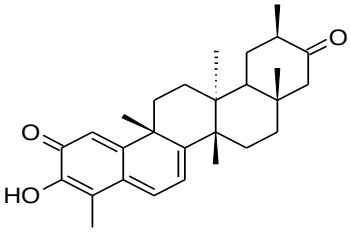

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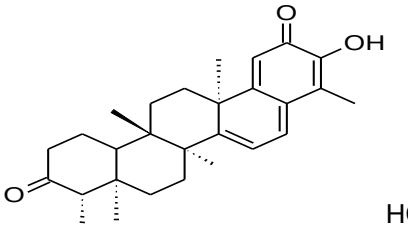

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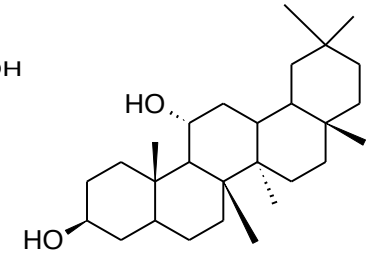

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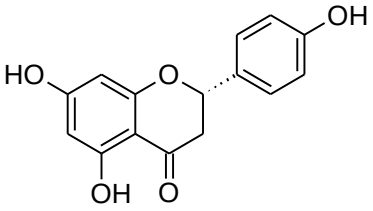

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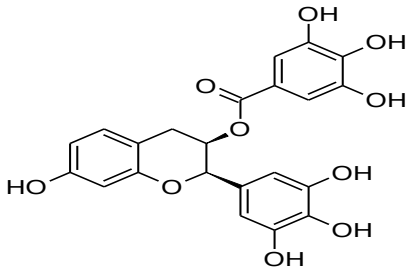

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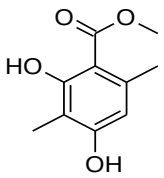

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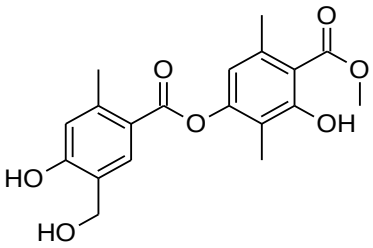

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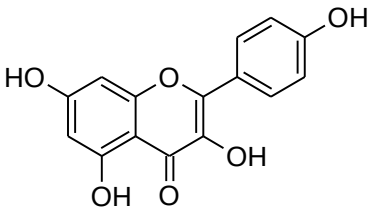

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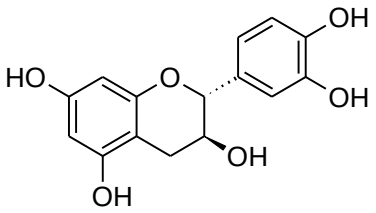

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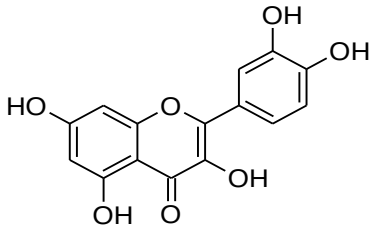

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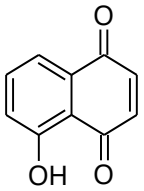

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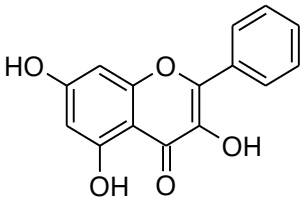

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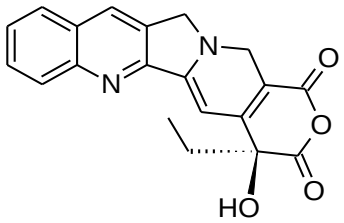

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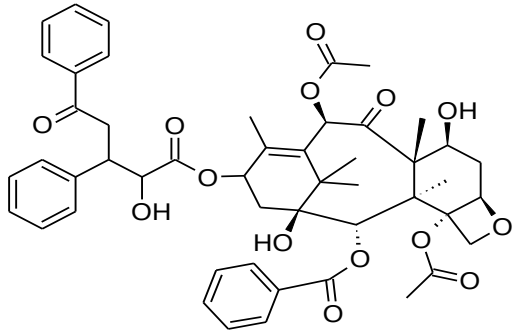

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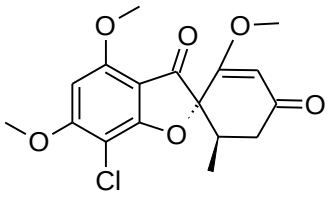

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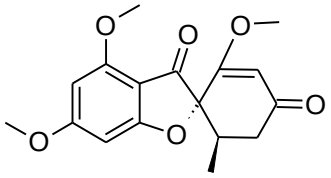

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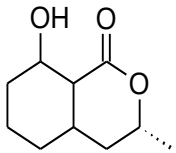

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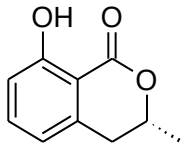

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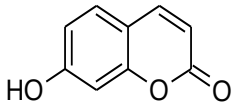

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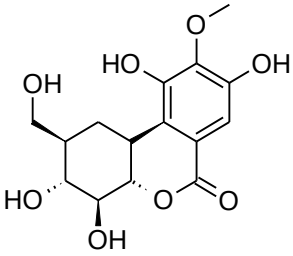

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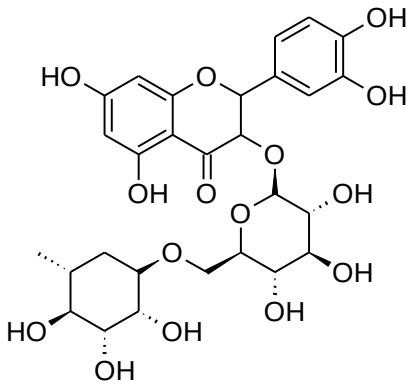

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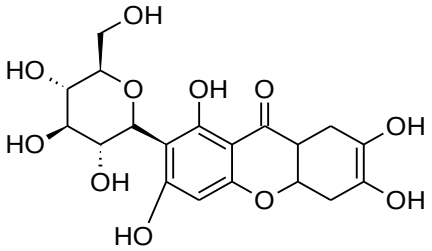

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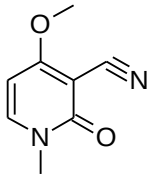

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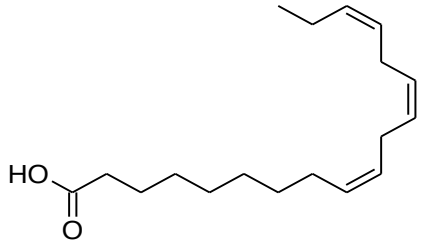

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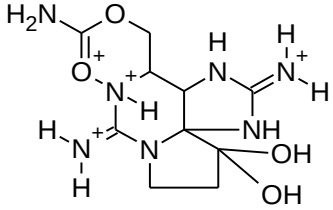

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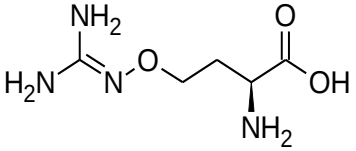

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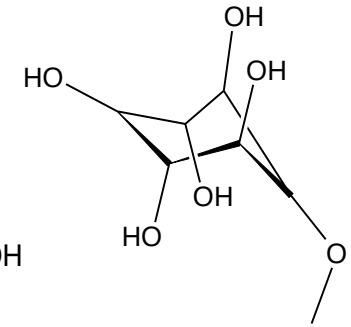

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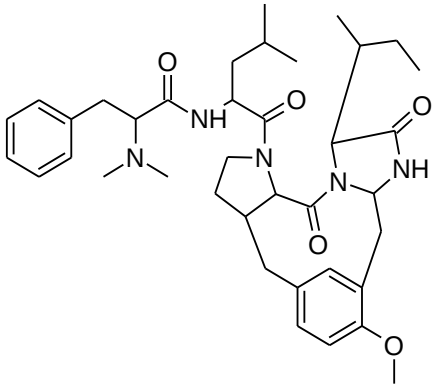

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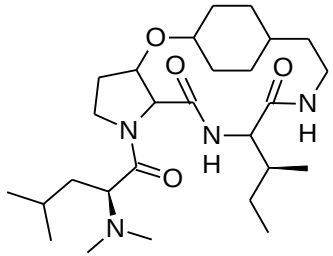

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Figure 1.1 Secondary compounds previously isolated from selected antiviral plants

1.4. Metabolomics

Modern-day research requires new technologies that enhance the duration, quality, and database of the research. Technologies such as metabolomics, proteomics, genomics and transcriptomics have contributed significantly to biological systems research. These technologies have allowed researchers to focus on studying the molecular profiles in cells or tissues, which are exposed to chemicals and thus may be used in drug discovery. Metabolomics is a technique that has been developed to assist in the biochemical analysis of complex mixtures of compounds, for fingerprinting and for predicting active metabolites in a group of plants of the same species or a single plant (Montanez et al., 2012). Metabolomics is essential in the investigation of phytoconstituents from medicinal plants but has a role in determining the nutritional values of different edible plants. It is thus a holistic, non-biased method for quality control of various foods, pharmaceuticals and disease biomarkers (Newgard, 2017).

Nuclear Magnetic Resonance (NMR) based metabolomics is a technique that allows both qualitative and quantitative analysis of metabolites. It is a preferred instrument in metabolomics because it provides rapid analysis and easy sample preparation although its major drawbacks include low sensitivity and sometimes overlapping peaks (Verpoorte et al., 2008). However, the low detection rate of the NMR can now be resolved by using a higher field magnet with cryogenic probes that aid in amplifying the signal and resolution (Wishart, 2008). To resolve the overlap in spectral peaks, different NMR spectrometric analyses may be performed, as for instance a two-directional *J*-resolved (JRES NMR) (Huang et al., 2015), a diffusion ordered (DOSY) (Novoa-Carballal et al., 2011), or ^{1}H-^{1}H/^{1}H-C^{13} homo- or heteronuclear (Alexandri et al., 2017). Another technique to supplement the NMR analysis is to use LC-MS to compliment the NMR and this may be an ideal technique which will accelerate metabolites identification and characterization since LC-MS is highly sensitive and capable of fragmenting metabolites to obtain precise structures. Metabolomics carried-out through NMR coupled with multivariate chemometric analysis can better assist in understanding the nature of metabolites that play a significant role in the discrimination of samples in terms of their concentrations and structural differences (Emwas et al., 2019). In a chemometric analysis, the use of multivariate statistical tools is employed to compare patterns and intensities of metabolites detected by the NMR (Trygg and Holmes, 2007).

This study aimed to utilise metabolomics using the ^{1}H-NMR in combination with multivariate data analysis to search for anti-RVFV metabolites from 20 South African medicinal plants pharmacologically known to possess antiviral properties. This is the first study to investigate and document the anti-RVFV activity of medicinal plants.

1.5. Objectives

- To determine the efficacy of twenty selected plants as potential crude drugs to reduce the RVF-viral induction cytopathogenic effect in virus infected Vero cells
- To determine variations in metabolite profiles of 20 medicinal plants used for viral inhibition
- To correlate the metabolite profiles of selected plants extracts to their inhibitory activity of RVFV
- Annotate and identify secondary metabolites responsible for the activity by means of ^{1}H-NMR-based metabolomics coupled with multivariate statistical analyses and UHPLC-MS analysis
- Asses the suppression of radicals by the most active plant extracts using non-cell-based and cell-based assays

1.6 Hypothesis

1. Pharmacologically-based selection of plant species with antiviral activity will exhibit significant *in-vitro* anti-RVFV activity
2. ^{1}H-NMR-based metabolomics coupled with multivariate statistical analyses and UHPLC-MS can annotate and/or identify anti-RVFV metabolites from selected medicinal plants.

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Chapter 2

Epidemiology, pathogenesis, transmission cycles and reactive oxygen species in Rift Valley Fever virus

2.1 Introduction to the epidemiological perspective of Rift Valley Fever virus (RVFV)

Human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV and HCV, respectively), coronaviruses, influenza (seasonal, pandemic), smallpox, viral haemorrhagic fevers (Ebola), dengue virus, and chikungunya virus are viral pathogens which have affected humans and animals for many year and have resulted in high number of morbidities. One possibility that makes viral pathogens to thrive is their mechanism of transmission. The transmission mechanisms of these viruses have been outlined and among others, they include direct contact, aerosol and sexual transmission. Other viruses require vectors to successfully transmit viruses to a new host, for instance, arboviruses require mosquitoes, biting midges, phlebotomine flies and ticks. Viruses transmitted by mosquitoes are becoming increasingly fatal with diseases like encephalitis, malaria, haemorrhagic syndrome and neurological disorders. Arthropod-borne viruses are prominent hematophagy transmitted diseases in Africa with malaria being the foremost arthropod-borne disease in southern Africa.

The RVFV is a tri-segmented genomic mosquito-borne pathogen which belong to the genus *Phlebovirus* of the family Phenuiviridae. It affects both humans and animals resulting in haemorrhagic fevers (Islam et al., 2018). RVFV is characterized by fatigue, anorexia and abortion and death in animals (Anyangu et al., 2010; Ikegami and Makino, 2011). In humans, most infections are self-limiting febrile illness ranging from a moderate fever to headaches, but severe cases can progress to sequalae, hepatitis, retinitis, haemorrhagic fever, encephalitis and mortality (Bird and McElroy, 2016). Patients show clinical symptoms including jaundice, diarrhoea, rash, low blood pressure, haemorrhage and gastrointestinal disorders have been reported (Kahlon et al., 2010). The virus is transmitted from one animal to another by mosquitoes of the *Aedes* or *Culex* and *Anopheles* genera and from mother to child during birth. Direct contact with fluids from an infected animal and mosquito bites is the pathway through which humans may be infected (Seufi and Galal, 2010). In 1930, the RVFV outbreak resulted in unusual mass abortions in pregnant lambs and high death rate in young animals in Kenya and for this reason, the first discovery of RVFV was made (Carroll et al., 2011). Since the emergence of RVFV in 1930, there have been numerous subsequent outbreaks in Africa and this includes an outbreak in South Africa between 1950 to 1975, which was the first fatal record in humans and another outbreak in 2010-2011 (Archer et al., 2013), Zimbabwe in 1978 (Grobbelaar et al., 2011), East Africa 1997-1998, Mauritania in 1998 and 2012 (Nabeth et al., 2001; Faye et al., 2007), Kenya, Somalia and Tanzania 2006-2007 (Sow et al., 2014; WHO,

2018), Niger in 2016 (WHO, 2016), South Sudan in 2018 (WHO, 2018) and Gambia in 2018 (WHO, 2018). The RVFV was firstly identified outside Africa in Saudi Arabia, Yemen 2000-2001 and China in 2016 (Madani et al., 2003; Liu et al., 2017). The Centres for Disease Control and Prevention (CDC, 2017) documented the outbreaks in Africa as shown in Figure 2.1. The most recent outbreak outside Africa was around November 2018 – March 2019, in Mayotte (France) were reported where 82 human infection cases were high with more prevalence of infections in the rural areas of Centre-West and North of the island (ECDC, 2019). The outbreak of the RVFV create a serious threat to human health, animals and has devastating effect on economy in terms of livestock production (Trevennec et al., 2012). The World Organization for Animal Health (OIE) has placed the RVFV in the list of notable diseases and classified it as a NIAID high-priority category A biothreat agent and a serious emerging public health concern (CDC; USDA).

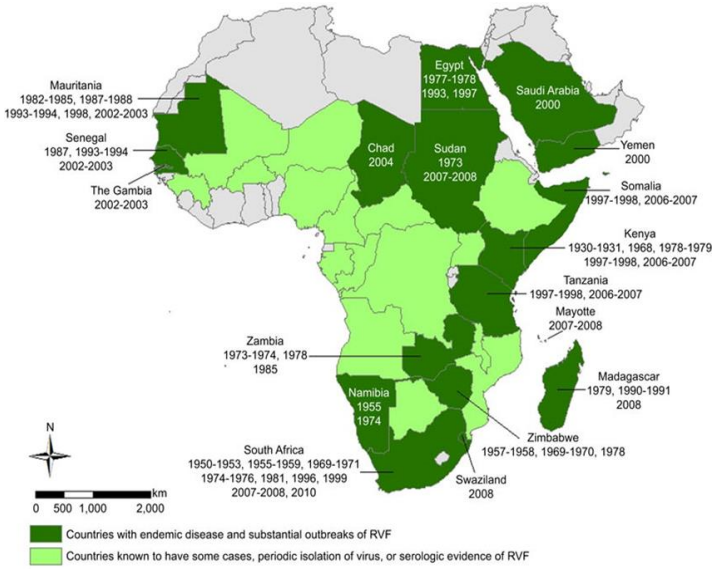

Figure 2.1 Latest outbreaks of RVFV (2006–2016) reported in Africa (Adopted from Rolin et al., 2013; CDC, 2017).

2.2 Pathogenesis and immune response to RVFV infection

With over 350 species in five genera, the Phenuiviridae family is regarded the largest viral family. They have a tripartite RNA strand (ssRNA) in the genomic structure and consist of viral RNA segments known as, small (S) medium (M) and large (L), which aid in the processes of virulence and infection (Figure 2.2) (Terasaki et al 2011; Kalveram et al., 2013).

The viral genome encodes for four proteins which are nucleoproteins (N) is vital in viral transcription, replication, packaging and mature virion architecture. The L-segment encodes two non-structural proteins which are conserved subjects essential for viral RNA synthesis. The M-segment produces the poly-translated mRNA which uses five translation start codons to make proteins that are processed by host signal peptidase. This process will then produce glycoproteins, which are arranged in the viral membrane into an icosahedral lattice and two non-structural proteins, NSm1 and NSm2. The glycoproteins Gn and Gc mediate the viral attachment and initiate fusion, respectively, whereas NSs serves as a host innate immune response inhibitor and NSm inhibits apoptosis induced by the virus (Ikegami and Makino, 2011; Kalveram et al., 2013; Dodd et al., 2013). The two proteins encoded by the S-segment, are nucleocapsid and the non-structural protein (NSs). The nucleocapsid protects the viral genome, while the NSs protein is virulence factor of RVFV and is crucial for evading the host innate immune response (Ly and Ikegami, 2016). However, N and L proteins are necessary for the replication and transcription. Moreover, to form the ribonucleoprotein (RNP) complex with the viral genome but NSm and NSs are not required for viral replication (Kalveram et al., 2013),

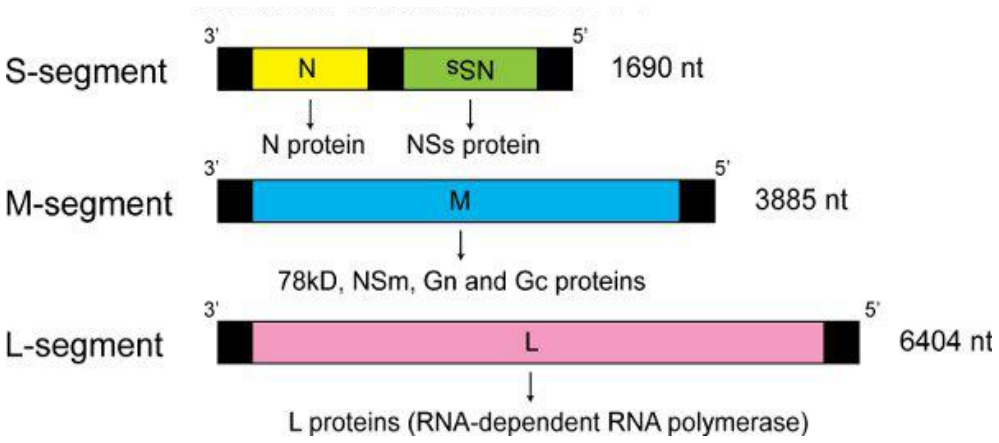

Figure 2.2 Schematic representation of a tripartite segmented RVFV genome containing of a Large (L), Medium (M) and S (small) segments. (Adopted from Kalveram et al., 2011, 2013)

Macrophages are important targeted cells for various diseases and serve as the first line of defence during immune pathogenesis. They are phagocytes found in different tissues and are crucial for stimulating immune responses. Macrophages can phagocytose cellular pathogens and may release antigens that the trigger antibody response through the MHC-II mode of response to the $CD4^+$ T-cells and $CD8^+$ cytotoxic T-cells (Murray and Wynn, 2011).

When the RVFV infects the macrophages, viral attachment and fusion into the host cells are mediated by the Gn and Gc receptors and the genomic material will be released into the cells (Spiegel et al., 2016). The RVFV will release NSs protein to suppress the host transcription and inhibit the secretion of antiviral interferon, nuclear factor kappa B, tumour necrosis factor (TNF), and the virus continues to replicate in the cytoplasm (McElroy and Nichol, 2012; Ly and Ikegami, 2016). Dodd et al., (2013) illustrated that the $CD4^+$ T cells are required to induce immunity towards RVFV encephalitis in mice injected with the attenuated RVFV strain without the NSs gene.

During the hepatic phase of infection, RVFV has caused apoptosis of hepatocytes, and viral antigens were found in the liver (Reed et al., 2012). In this case, the expression of the pro-inflammatory genes and pro-apoptotic gene were triggered in the liver of mice. RVFV virulent strain infected mice showed an increased expression of cytokine and chemokine such as IL-12 which induces IFN-γ from natural killer (NK) cells. This response is a TH1 which serves to reduce the virus (Jansen et al., 2011). The unregulated secretion of cytokines and chemokine leads to severe liver damage when animals are infected with the wild-type RVFV. At the late stages of infection, RVFV affects the central nervous system (CNS) and blindness and neurological defects may occur (Ikegami and Makino, 2011). The mechanisms of the virus to invade the CNS is not clear but one possibility is through neuronal penetration through infection of antigens released by macrophages and dendritic cells. Mice infected with RVFV showed that the virus can invade the brain via the central nervous system as the viral antigens were present in the brain, stem, and spinal cord (Smith et al., 2010).

2.3. RVFV transmission cycle

Mosquitoes and ticks are vectors that feed on blood meal primarily transmit arthropod-borne diseases among mammalian hosts (Amraoui et al., 2012). Transmission modes may be horizontal or vertical of which horizontal transmission is common for most arboviruses. When the virus genome is passed sexually from an infected male vector to a female vector is known as horizontal transmission. Other than sexual transmission, female vectors may pass the virus to mammals via hematophagy. In contrast, vertical transmission is when an infected female vector passes the virus to its progeny (Figure 2.3). Inside the vector, the virus must undergo replication in the gut tissue and the salivary glands before it can be transmitted. Infected

humans cannot transmit the virus to other humans, therefore humans are dead-end hosts (Bird and Pepin, 2010; McElroy, 2016; McMillen and Hartman, 2018).

Ecological aspects and climatic conditions such as rain, temperature, and humidity are important parameters in the epidemiology of RVFV. The prevalence of RVFV can be best associated with areas acquiring high rainfall, heavily flooded and high temperature, as these areas offer a conducive breeding place for mosquitoes. Most of the RVFV outbreaks have occurred frequently in 5-15 years periods following sever precipitation (Anyamba et al., 2001; Pepin et al., 2010; Danzetta et al, 2016). In Africa, rehydration of mosquito eggs from heavy rainfall leads to egg hatching, causing an increase in the progeny and may result in epizootic cycles (McMillen and Hartman, 2018). Dependence of the arboviruses to the ecological and climatic condition can be factor contributing to the establishment and transmission cycle of the viruses, however, unfavourable conditions lead to enzootic and interepizootic cycles in arboviruses (Lafferty and Mordecai, 2016).

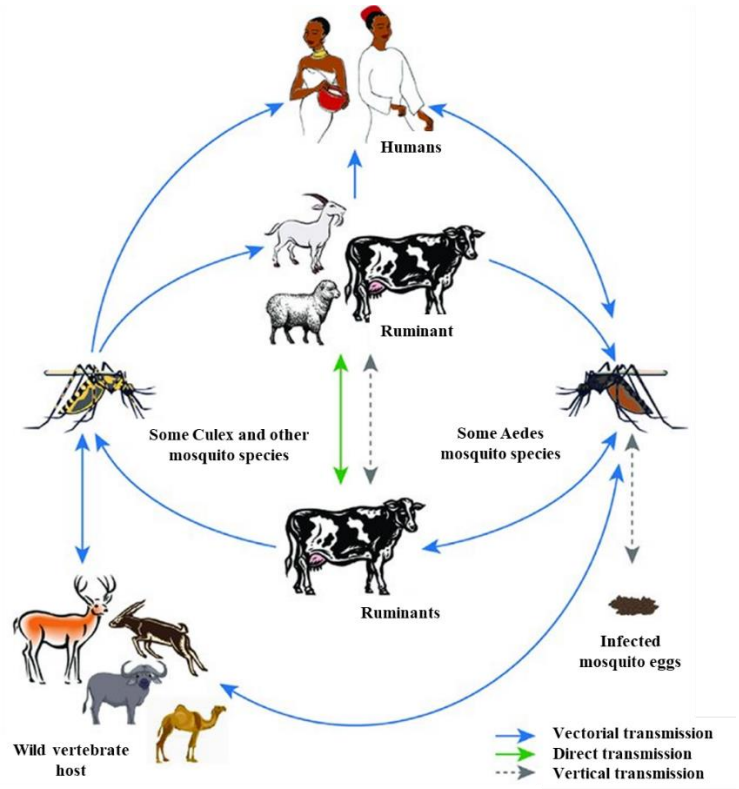

Figure 2.3 An illustration of RVFV lifecycle, vector transmission, direct and vertical transmission (Adopted from Balenghien et al., 2013).

2.4 Current RVFV treatments and developments

There are three approved classes of vaccines available currently used against RVFV for livestock and these are alternative vaccines, inactivated and live attenuated virus but since the discovery of RVFV, vaccines for humans are not licensed (Table 2.1) (Pepin et al., 2010; Boshra et al., 2011). Although there are vaccines available for the ruminants, there are limitations to their use in endemic regions. Kortekaas (2014), have documented the endorsement of Clone-13 in South Africa and Zimbabwe, whilst the MP-12 has been conditionally endorsed to be used in the United States (Table 2.1).

A few compounds tested using cell culture and animal models showed antiviral potency. The first antiviral being tested in models of phleboviral infection was a nucleoside analogue, Ribavirin. It has been tested against RVFV ZH501 in Vero cells and it exhibited an EC_{50} value of 80 μg/mL. The mouse model infection with a closely related phlebovirus, Punta Toro virus, subcutaneous (SC) daily administration of Ribavirin significantly reduced the virus replication and severity of symptoms with 100 % survival at 18.8 mg/kg. Favipiravir, a viral RNA polymerase inhibitor of influenza viruses (H1N1, H5N1 and H7N9) and avian virus (Furuta et al., 2014) showed inhibitory effects of RVFV cultured in Vero cells with an EC_{50} of 32 μM and in the hamster model protected 80% of SC infected animals with 30 pfu of RVFV ZH501 with 10 days. Another study concerning finding active anti-RVFV tested 10,000 compounds and two related compounds of 3,7-bis(dialkylamino)phenothiazine-5-ium skeleton termed 6051 and 7007 were identified. These compounds showed plaque reduction against RVFV and Lassa Fever virus with EC_{50} values < 0.4 μM and < 5 μM, respectively.

Suramin is an inhibitor of trypanosomiasis and onchocerciasis used in Africa, which acts on the early stages of the RVFV lifecycle. Suramin has been shown to disrupt the vRNP complexes on entry late stages of RVFV pathogenesis. When tested *in-vitro* on RVFV cultured in HEK 293T an EC_{50} value of 22.3 μM was observed. Other molecules such as Rapamycin (BALB/c mice), Bortezomib (H2.35 cells) and Sorafanib (Vero cells) showed potency against RVFV with EC_{50} values of 11 μM, <0.01 μM and 6.4 μM respectively (Atkins & Freiberg, 2017). Recently, Benzavir-2, which has shown potency by inhibiting the herpes simplex virus (type 1 and 2), human adenovirus and was tested against RVFV. This study demonstrated dose-dependent inhibition of RVFV RNA expression with an EC_{50} value of 1.7 μM (Islam et al.,

2018).The lack of approved treatments for RVFV poses a serious challenge and a threat of the emerging viral infectious disease. Therefore, research and development of safe, effective new antiviral agents with less adverse effects are required for the management of viral infectious diseases and their related co-morbidities.

Table 2.1 RVFV vaccines with their adverse effects (Boshra, Lorenzo and Brun, 2011)

Vaccine type	Limitations	References
Live attenuated		
Smithburn	Teratogenic in fetuses, Abortifacient in livestock	Smith et al., 2018
MP-12	Teratogenic in pregnant ewes, Abortifacient in ewes and lambs	Hunter, Erasmus, and Vorster, 2002;
Inactivated		
Formalin-inactivated, NDBR103 and TSI GSD 200	Neutralising antibody titre	Pittman, 2017
Alternative		
Clone-13	Overdose may cause fetal infections, malformations, and stillbirths	Makoschey et al., 2016
	Neurological disease in mice	Dodd et al., 2013
Recombinant protein	Neutralising antibody titre	Heise et al., 2009
DNA Vaccines	Weak protective host immune responses	Faburay et al., 2014
Virus-like particles	Opposition against the use of genetically modified products	Mansfield et al., 2015

2.5 Reactive oxygen species in RVFV infection

Upon RVFV infection, cytokines and other cell mediators are released to fight against the infection. this reaction limit viral proliferation, while it also causes immunopathology. During infection, oxidative stress in host cells is produced by accumulation of reactive oxygen species (ROS) as a result of an innate immune response, cellular metabolism. However, an inconsistencies amongst antioxidants and oxidants with an increase of oxidants, results in tissue damage, DNA damage, inflammation response and cell death (Ivanov et al., 2017). Reported studies showed that apoptotic responses in liver cells were a result of RVFV infection (Reed et al., 2012) and modulation of the host oxidative stress responses by viral proteins have facilitated its replication. In its defence, the immune system needs to release antioxidants to suppress or neutralise the effects of ROS. In normal circumstances, the antioxidative defence is made to turn off, but during the detection of oxidative stress, an activation of genes sequence results to detoxify and for cytoprotection. These genes activate the release of antioxidative inflammatory mediators such as nuclear factor erythroid2p45 (Nrf2) to suppresses the viral-induced oxidative stress. However, viruses have evolved ways to manipulate Nrf2 activities (Davinelli et al., 2014) and continue replicating in cells. Survival of the virus in cells further overwhelms the immune system by creating an imbalance between antioxidants and pro-oxidants. Nrf2-activators is found in beverages and vegetables which promote good health and these may include epigallocatechin-3-gallate, quercetin, pterostilbene, cafestol, sulforaphane, curcumin, resveratrol and carnosol (Balstad et al., 2011). Lii and Yao, (2013) demonstrated the inhibition of tert-Butyl hydroperoxide (tBHP)-induced oxidative stress through expression of the extracellular Signal-Regulated Kinase 2/ nuclear factor erythroid 2-related factor 2 (ERK2/Nrf2) pathway by phytochemicals such as flavonoids: luteolin and apigenin. This is the reason for an enhanced of defence against oxidative stress associated neurodegenerative diseases such as diabetes mellitus, Alzheimer's disease, Parkinson's disease, atherosclerosis diseases, age-related degeneration diseases, rheumatoid arthritis, and cancer.

Other immune pathways involved in antiviral properties are manipulated and exploited by viruses to replicate and persist within their hosts, therefore exacerbating the disease progression which includes the stimulation of NF-κB protein (Zhao et al., 2016). Interferon (IFN-γ) has been reported to induce antiviral enzymes like guanylate-binding proteins, dsRNA-specific adenosine deaminase and dsRNA-regulated protein kinase R (PKR). However, HIV-1 has been reported to suppress the induction of IFN-γ human dendritic cells

and macrophages, leading to viral persistence (He, Tran and Sanchez, 2019). A negative influence of gene expression can be regulated by trans-activator of transcription (Tat) binding to a promoter sequence and the residence of Tat in the active site of NF-kB may lead to downregulation of c-Rel expression (Clark, Nava and Caputi, 2017).

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Chapter 3

Cytotoxicity, antiviral and radical scavenging activity of selected antiviral medicinal plants

3.1 Introduction: Rift Valley Fever Viruses

Arthropod-borne viruses (arboviruses) have a history of emerging to infect humans and animals. These arthropod-borne viruses are namely zika viruses, yellow fever, dengue, chikungunya and, rift valley fever (Rautenbach, 2011). They are primarily transmitted by *Aedes aegypti* mosquitoes, and can spread from animal to humans (Thongsripong et al., 2018; Portillo et al., 2018). Rift Valley fever is an emerging zoonotic, mosquito-borne viral disease that primarily affects livestock and humans (Pepin, et al., 2010). Infection with RVFV present many symptoms ranging from asymptomatic to a haemorrhagic fever and severe symptoms may include vascular permeability, coagulation defects and fatal hypovolemic shock (Pepin, Ouloy, et al., 2010). Re-emergence of these viruses in tropical and subtropical regions have posed challenges in the health care system of many developing countries as there has been limited treatments for these viruses (Rautenbach, 2011; Sigfrid et al., 2018). This has warranted for more research on finding antiviral agents that can be used in management and treatment of RVFV infected humans and animals.

Medicinal plants have portrayed an important part as a foundation of many therapeutic agents (Newman and Cragg, 2006) and the increasing use of these plants worldwide, warrants for more scientific validation of their efficacy, safety and quality (Segall and Barber, 2014). Faced with the fast-growing demands for therapeutic agents, synthetic medicine has shown to possess harmful adverse effects such as toxicity and, in many cases, they are unable to bind to certain receptor systems as compared to natural products. However, traditional medicines are gaining greater appreciation because of their enhanced compatibility with the human body and reduced side effects (Commentary et al., 2017). Cytotoxicity evaluation is the crucial step in determining possible toxicity of plant extracts, in the development of new therapeutic agents (pharmaceutics, cosmeceuticals and nutraceuticals). Cytotoxicity assays indicate the potential of possible therapeutic agents to cause cell death and tissue injury. In scientific research of the efficacy of medicinal plants, cytotoxicity tests are very important as they help in the determination of the future of plants for medicinal use (Segall and Barber, 2014).

Cytotoxicity assays are a quick, easy to execute and inexpensive way to assess the effectiveness of a compound on normal human cells. The MTT assay is the most popular technique of measuring cell viability or cell mortality. This method was applied in this study

to determine the cytotoxicity of twenty medicinal plants, which were previously pharmacologically investigated and reported to possess antiviral activity. The application of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into living cells results in the reduction of the dye by the dehydrogenases enzymes, to form an insoluble purple formazan crystals, which is indicative of cell viability (Tonder et al., 2015).

The cellular metabolism produces acceptable levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), however, the overproduction of these inflammatory mediators results in oxidative stress which is implicated in the pathogenesis of many viral and non-viral diseases (Ivanov et al., 2017). Research studies have reported viral infected patients who are under chronic oxidative stress and these include infection with the HIV, rhinovirus, hepatitis C virus (HCV), and arthropod-borne viruses (Comstock et al., 2011; Ivanov et al., 2016; Ivanov et al., 2017; Couret et al., 2017). ROS is vital in cell signalling, mitochondrial biogenesis, immunity, cell proliferation and differentiation, but the accumulation of ROS is central in cellular lipid peroxidation and DNA damage (Saldaña et al., 2017). The various antioxidative enzymes are expressed by viral infection, to ameliorate ROS damage in host cells, therefore, suppression of ROS to normal required levels will have antiviral activity and cell death might be avoided.

Given the high mortality rate related to severe infection of RVFV, there is a pressing need to implement new therapeutic treatments to manage RVFV infections (Rautenbach, 2011; Sigfrid et al., 2018). This implementation will need to achieve a rapid response and a state of readiness to an epidemic outbreak. Determination of acceptable non-toxic doses of the extracts on African green monkey kidney (Vero) cells was performed for the subsequent study of their antiviral activity using the MTT cytotoxicity assay. Furthermore, this investigation seeks to evaluate the non-cell-based antioxidant scavenging potency on DPPH and $ABTS^{+}$ radicals. Additionally, we aim to conduct a cell-based antioxidant activity which measure the suppression of LPS-induced ROS by selected plant extracts.

3.2 Materials and Methods

3.2.1 Sample preparation and extraction

Fresh leaves were desiccated at 25 °C in the dark, after which, they were grounded to a fine powder using a grinding mill (IKA™ MF10 Mill). Fifty grams (50 g) of the pulverized plant material were extracted twice with 50 mL of 50 % aqueous methanol by shaking in room temperature for 24 h. Samples were filtered using a Buchi® filtration system and concentrated under high vacuum (EZ-2plus GeneVac™ evaporator, USA).

3.2.2 Cell cultures and cytotoxicity assay

The cytotoxicity assay was done using the (MTT) assay following a method by Mosmann, (1983) with slight modifications. Vero cells viability and growth was sustained in Dulbecco's modified Eagle's medium (DMEM; Gibco) with added mixture of 1 % penicillin/streptomycin and 10 % fetal bovine serum (FBS) in culture flasks and incubated at 37 °C and 5% CO_2. When cells reached 85 % confluency, cells were detached using 2 % trypsin and cell count was performed using an automated desktop cell counter TC20™ (BIO-RAD). Hundred microliters of cells (1 x 10^4 cells/well) were added into 96-well plates and grown overnight at 37 °C in 5 % CO_2-incubator to allow cell adaptation and attachment. Different extracts treatment was administered with varying concentrations ranging from 0 to 1000 mg/mL. Doxorubicin and 5 % dimethyl sulfoxide (DMSO) were used a positive and negative control, respectively. The MTT solution (20 µL of 5 mg/mL in Phosphate-buffered saline - PBS) was added in all the wells and incubated for 4 h. to dissolve the formazan crystals, DMSO (100 µL) was added. A yellow tetrazolium salt (MTT) is reduced to an insoluble deep purple formazan crystalline by metabolically active cells. The optical density was measured at 540 nm using an ELISA microplate reader. Percentage of cell viability was calculated using the formula below.

$$\textbf{\textit{Cell viability}}\ (\%) = \left({}^{A_1}/_{A_0}\right) \boldsymbol{x}\ \mathbf{100}$$

Where symbols A_1 and A_o expresses the absorbance treated and control cells, respectively. Cytotoxicity was presented as 50 % inhibitory concentration (IC_{50}) of screened plant extracts that reduced 50 % viable cells compared to control cells.

3.2.3 Antiviral activity assay

The antiviral activity of plant extracts against RVFV was determined on Vero cells using the cytopathic effect (CPE) inhibitory assay. The principle of this assay is to inhibit the formation of CPE, which is indicative of antiviral activity since viruses induce CPE. Vero cells were maintained in DMEM (Gibco) supplemented with 10 % FBS and a mixture of 1 % pen/strep using the filtered T25 culture flasks. Adhered cells were detached after incubated at 37 °C and 5 % CO_2. by adding 2 % trypsin and cell count was achieved using an automated cell counter (TC20™ BIO-RAD). RVFV suspended in DMEM was mixed with one non-toxic concentration (100 µg/mL) of each extract in sterile capped vials. The inoculum was incubated overnight at room temperature. The extract-virus suspension (100 µL) was serially diluted (10^{-1}–10^{-8}) with media in 96 well plates and 100 µL of cells was added in all the wells and incubated for 7 days until CPE appeared. The determination of CPE on cells was executed on a light microscope and comparing treated and control cells. The Tissue Culture Infectious Dose ($TCID_{50}$) values were calculated using the Spearman and Kärber algorithm method. Consequently, less CPE's were considered as an indicator of antiviral activities of the extracts. Virus + cells only (positive control) and medium + cells only (negative control) were included in this experiment as controls.

3.2.4 Real-Time Cell Analyzer (RTCA)

The effects of RVFV and the protective ability cells by extracts was monitored by the real-time cell analyser RTCA xCELLigence DP system (ACEA Biosciences). Briefly, 100 µL of cell culture media was added to each well of the plate (ACEA Biosciences Inc). The plates were connected to the RTCA system to calibrate, optimize and to obtain background impedance readings in the absence of cells within 2 minutes. After this reading, the media was aspirated, then 1 x 10^4 cells/well were seeded in all the wells and incubated for 1 h at 37 °C under 5% CO_2, after which the plates were connected onto the RTCA system inside the incubator for continuous impedance recording for 24 h. The media was aspirated and 100 µL of the virus with extract (100 µg/mL) was added and the plates were allowed to stand for 2 h to allow adsorption of the suspension. Control wells containing cells only and virus infected cells without extracts were included in this study. The experiment was allowed to run for 170 h with cell index (CI) values measured every 15 min on the E-plate 16 re-connected onto the RTCA xCELLigence system.

3.2.5 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity

The antioxidant assay on DPPH radical is based on the reduction of a free radical (DPPH) by a molecule which can donate protons. The assay was done using an adapted method by Rangkadilok et al. (2007). Twenty microliters (20 µL) of 1.0 mg/mL extracts was pipetted into 200 µL of methanol, added to the first top wells. The solutions were serially diluted onto the remaining wells of the 96 well plate, which contained 110 µL of methanol. Ninety microliters of 0.1 mM methanolic-DPPH was added to all the wells. The extract concentrations ranged from 0.781 to 100 µg/mL. The plates were allowed to incubate at 25 ºC for 30 min. Thereafter, the absorbance was measured at a wavelength of 517 nm. Ascorbic acid (vitamin C) was used as the positive control at the same concentrations as the extracts. The extracts effect on scavenging the DPPH-radical was calculated using the below equation.

$$\boldsymbol{DPPH\ Scavenging}\ (\%) = \left({}^{\boldsymbol{A_o - A_1}}/_{\boldsymbol{A_o}}\right)\ \boldsymbol{x\ 100}$$

With A_0 and A_1 representing the absorbances at 517 nm of the DPPH in the absence and presence of an antioxidant respectively.

3.2.6. Assessing the ABTS$^+$ (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging assay

The radical scavenging inhibitory potency of plant samples was measured by the ABTS$^+$ radical cation decolorization assay. ABTS$^+$ cation radical was produced by the reaction between ABTS$^+$ (10 mg) and potassium persulfate (2 mg) in water. The mixture was preserved at room temperature for 12-16 h before use in the dark. after which the ABTS$^+$ mixture (1 mL) was diluted using 60 mL of methanol. The assay was performed following a technique as defined in section 3.2.5. The inhibition percent of the absorbance measured at 734 nm (Prior, Wu & Schaich, 2005) was calculated using the formula below and measurements were done in triplicates.

$$\textbf{\textit{ABTS Scavenging}}\ (\%) = \left({A_o - A_1}/{A_o}\right)\ \textbf{x 100}$$

The symbols A_0 and A_1 represents the absorbance of $ABTS^+$ radical and methanol and the absorbance of $ABTS^+$ radical and sample extract, respectively. The inhibitory percentage of $ABTS^+$ was presented by the effective concentration (EC_{50}) value, which is the concentration desired to reduce the radicals by 50 %.

3.2.7 Measurement of LPS-induced reactive oxygen/nitrogen species

The assessment of ROS was done using Vero cells following the method by (Chen et al., 2013; Wu et al., 2015) with minor modifications. In 96 well plates, cells were seeded at 1 x 10^4 cells/well and were incubated in 5 % CO_2 at 37°C for 24 h. After 24 h incubation, extracts (100 µg/mL) were added and stimulation with ROS at non-toxic doses of 1 µg/mL LPS (Wu et al., 2015), serving as the positive control. After incubation, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) 10 µM, was added and further incubated for 30 min in the dark. Measurement of the fluorescence was done at 485 and 535 nm excitation and emission, respectively, on a microplate reader (VarioSkan Flash, Thermo Fisher Scientific, Finland).

Similarly, cells cultured on TPP-Clipmax 10 cm² (PromoLab Pty Ltd, Separations) fitted with a filter screw cap were subjected to similar treatments as described in assessment of ROS above. The glass slides with cells were detached from the Clipmax chamber and the cover slip was then mounted on a glass slide. Images were computed on a laser scanning microscope (LSM 710, Zeiss, Germany).

3.2.8 Nitrite concentrations as a measure of reactive nitrogen species

The supernatant of Vero cells was used to measure the presence NO production in the presence of extracts and LPS. Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) was added to supernatants harvested from the above-mentioned ROS experiment. The optical density was measured at 550 nm in a microplate reader (VarioSkan Flash, Thermo Fisher Scientific, Finland) after 10 min incubation.

3.3 Statistical analyses

Experiments were done in triplicate or in quadruplets where necessary with three independent assay repeats and results are presented as mean ± standard deviation (SD). GraphPad Prism software (Version 8.0) was used to determine the one-way (ANOVA) Noteworthy variances between the means of treated and control was done using the Duncan's multiple comparison t-test. Differences were significant at * $p \leq 0.05$; ** $p \leq 0.01$.

3.4 Result and discussion

3.4.1 Cytotoxicity results

The cytotoxicity assay was executed to establish the concentration range of extracts be tested in the non-toxic range for Vero cells for the antiviral activity. The United States National Cancer Institute (NCI) prescribes that crude extracts are safer at lethal concentrations (LC_{50}) greater than 20 µg/mL after 48 h incubation with cells (Abdel-Hameed et al., 2012). The cytotoxicity lethal concentrations (LC_{50}) of the tested plant extracts are presented in Table 3.1 in comparison to untreated cells. All plant extracts displayed a varying degree of non-toxic behaviour with LC_{50} range of 82.0 – 400.6 µg/mL. The cytotoxic concentrations that suppressed 50 % of cell growth were all above 20 µg/mL, which denotes that plant extracts are safer at given LC_{50} values (Table 3.1). A dose-dependent behaviour of cell viability in response to different concentrations of the extracts was observed (Appendix, Figure 6.1). Cell viability of less than 40 % was observed at the maximum concentration tested and as the concentrations decrease more viable cells observed. It is worth noting that *Ricinus communis* exhibited a significantly low LC_{50} of 82.0 µg/mL, which was expected since the plant's toxicity to humans and animals was previously reported with ricin and ricinine being the toxic constituents (Dorner and Dorner, 2011; Souza et al., 2018). In addition, *Elaeodendron croceum* was expected to be among the extracts with low LC_{50} values since its toxicity has been reported. Digitoxigenin-glucoside from *E. croceum* extracts was evaluated for its cytotoxic effects on Vero cells using the colorimetric (MTT) assay. Results from this study showed that digitoxigenin-glucoside possess cytotoxic effects with a recorded 20% cell viability at a concentration of 25 µg/mL (Prinsloo, et al., 2010).

Other *E, croceum* constituents namely 20-hydroxy-20-epitingenone and tingenone tested against HeLa, MCF-7 and SNO cell lines exhibited toxicity with IC_{50} values ranging

between 2.5 µM to 0.4 µM (Yelani et al., 2010). *Aloe ferox*, well renowned for its healing properties and commercialised for its use in pharmaceuticals, cosmeceuticals and nutraceuticals was the second extract that showed a moderately low LC_{50} value of 151.8 µg/mL. Celestino et al., (2013) investigated the toxicity of *A. ferox* resin tested at 50, 100 and 200 mg/kg. The results showed that *A. ferox* resin induced a significant increase in gastrointestinal motility with an ED_{50} of 19.01 mg/kg and they concluded that *A. ferox* is a laxative and non-toxic.

3.4.2 Antiviral activity results

The virus-induced cytopathic effect (CPE) in Vero cells that were infected with tenfold serial dilutions combined virus and extracts, was determined using the inverted microscope and real-time monitoring assay which allowed the quantification of viable cells and virus infectivity. The virus-induced CPE were monitored microscopically after 7 days when pronounced CPE has formed, and CPE scores were allocated based on the severity of monolayer deformation and the $TCID_{50}$ values were calculated. At day 7, a pronounced CPE and detachment of cells were observed in wells infected with 10^2 to 10^6-fold dilutions of the virus in the control experiment. To confirm the virus-induced cytopathic effect, 20 µg/mL of MTT solution was added after assessing the CPE followed by isopropanol to dissolve the formazan crystals. The MTT assay showed that cells with high viral titre had a clear light-yellow MTT solution which signifies cell death, while cells containing low viral titre and less CPE had dark purple MTT solution demonstrating cell viability and extract-induced viral death. Hence, the development of formazan crystals in viable cells allows for an indirect detection of the RVFV-infected cells.

The *E. croceum* and *Artemisia afra* extracts screened at 100 µg/mL showed potency by extensively reducing the number of $TCID_{50}$ up to 10^2 log of the RVFV infectivity followed by *Adansonia digitata* with a reduction of up to 10^4 log. An average potency compared to *E. croceum* was observed in *Elaeodendron transvaalensis*, *Euclea natalensis*, *Helichrysum aureonitens*, and *Sutherlandia frutescens* which lowered the RVFV viral load when 100 µg/mL of the extracts was applied to the virus (Figure 3.1). Eight extracts (*A. afra*, *A. digitata*, *E. natalensis*, *E. croceum*, *E. transvaalensis*, *E. elephantina*, *H. aureonitens*, *S. frutescens*) with best antiviral activity were select for further investigation including the radical scavenging

activity by means of the DPPH and $ABTS^{+}$ assays, and phytochemical analysis by UHPLC-qTOF-MS.

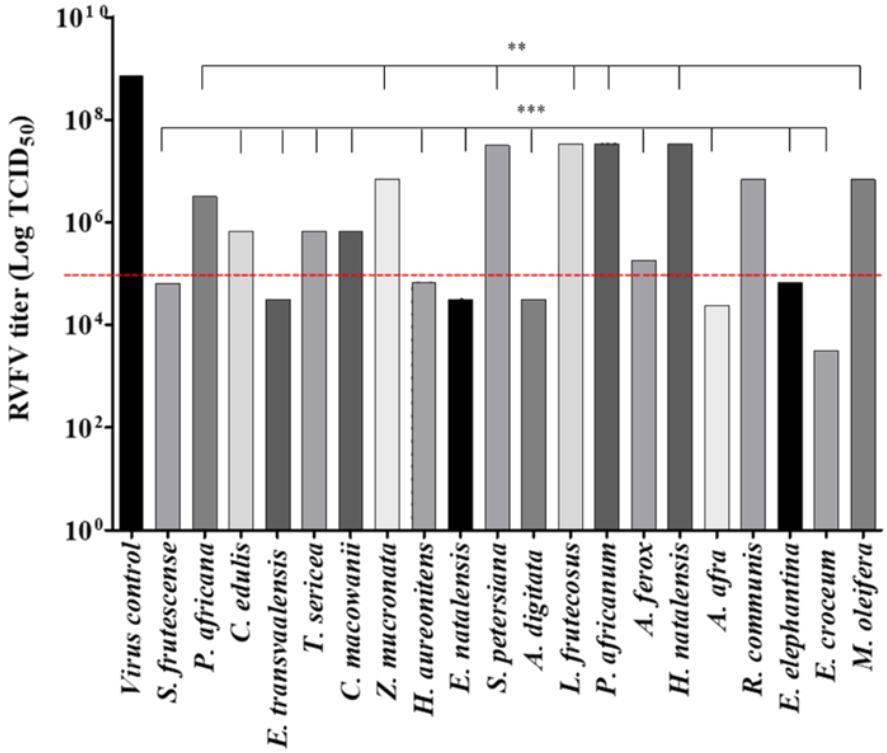

Figure 3.1 Illustration of the results of the plant extracts on the RVFV Tissue Culture Infectious Dose ($TCID_{50}$). The red scattered horizontal line represents the limit of viral susceptibility/resistance to extracts. The data symbolises the means from three independent experiments. A one-way ANOVA and Duncan's test for multiple comparisons were used for statistical analysis (** $p < 0.01$; *** $p < 0.001$). Experiments were conducted in triplicated.

Table 3.1 Non-toxic LC_{50} (µg/mL) values; DPPH and $ABTS^{+}$ scavenging activity effective concentration EC_{50} (µg/mL) of eight extracts with the best $TCID_{50}$ results.

Plant names	**LC_{50} (µg/mL)**	**DPPH EC_{50} (µg/mL)**	**$ABTS^{+}$ EC_{50} (µg/mL)**
Adansonia digitata	291.5	**4.64**	**5.04**
Artemisia afra	151.8	20.41	16.39
Aloe ferox	330.3	NE	NE
Carissa edulis	400.6	NE	NE
Crinum macowanii	389.4	NE	NE
Elaeodendron croceum	394.4	**6.00**	**4.12**
Elaeodendron transvaalense	336.9	11.64	15.00
Elephantorrhiza elephantina	225.2	**6.54**	**7.40**
Euclea natalensis	214.3	**5.30**	**5.00**
Helichrysum aureonitens	305.2	**8.25**	11.40
Heteropyxis natalensis	259.7	NE	NE
Lobostemon fruticosus	313.4	NE	NE
Moringa oleifera	271.6	NE	NE
Peltophorum africanum	332.5	NE	NE
Prunus africana	249.1	NE	NE
Ricinus communis	82.0	NE	NE
Senna petersiana	328.7	NE	NE
Sutherlandia frutescens	301.9	32.20	42.30
Terminalia sericea	232.2	NE	NE
Ziziphus mucronata	271.6	NE	NE

LC_{50}: lethal concentration of extracts to 50 % of Vero cells; EC_{50}: concentration of extracts that scavenge 50 % of radicals; NE: not evaluated. Positive control, Doxorubicin LC_{50} = 10 µM, Ascorbic acid EC_{50} values for DPPH and $ABTS^{+}$ assays were 2.50 µg/mL and 2.30 µg/mL, respectively. Bold values indicate extracts which are considered potent.

3.4.3 Antiviral activity by Real-Time Cell Analyzer (RTCA)

Eight extracts that revealed the greatest potential anti-RVFV were selected for the RTCA assisted antiviral viable cell monitoring. This technique records impendences of viable cells over time in the presence or absence of the virus and extracts incubated with the cells. The extracts of *E. croceum* and *A. afra* could effectively prolong the proliferation of Vero cells in a viral-extract co-culture. Cell proliferation was observed between 96 h to 150 h and a rapid decrease in cell index (CI) was experienced thereafter. This signifies that the extracts are inhibiting the progression of the virus-infection that causes the cytopathic effects and cell death. Other extracts including *A. digitata*, *E. transvaalensis*, *E. natalensis* and *E. elephantina* showed good viral inhibition with a rapid decrease in CI at approximately 140 h and *H. aureonitens* and *S. frutescens* moderate viral inhibition (Figure 3.2). Observed RTCA results correlates with antiviral activity assessed by tissue culture infectious dose.

The use of technology for monitoring of the viral-induced cytopathic effects by RTCA indicated a steady increase in cell index (CI) values within 24 h prior to treatment and post-treatment which is indicative of cell viability. Post-treatment, the slight increase in CI values continued up to 90 h and this can be best correlated to the viral incubation and adaptation in cells. Only after 90 h, a proliferative phase where rapid cell growth in extract-viral treated cells was observed, with only viral infected cells (control) having decreased CI values after 110 h, which is indicative of viral-induced cell death (Figure 3.2). Cells then went to a stationary phase between 120–140 h and then a steady drop in CI values was experienced, which reflects morphological changes and loss of viability. In summary, the cell proliferation was short in viral infected cells (control cells) as compared to the plant extract treated cells. Therefore, this may suggest that the RTCA xCELLigence system quantified the cyto-protective effects of extracts against the RVFV.

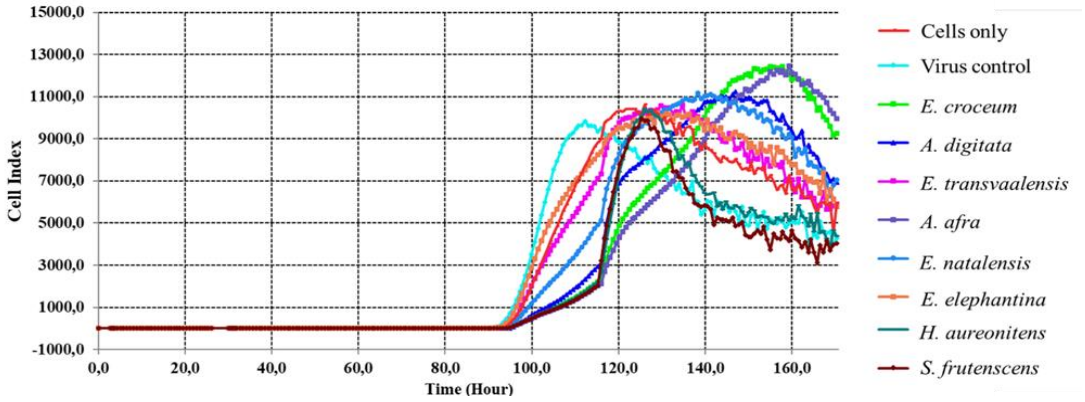

Figure 3.2 The antiviral effects and cell viability in response to viral infection and/or extract treatment using the xCELLigence RTCA analyser. Coloured curves represent the Vero cells (control), viral infected cells and extract (100 µg/mL) treated viral infected Vero cells.

Viral infections are known to activate the production of ROS and RNS species to abnormal cellular levels. Some studies emphasise that lowering of the ROS/RNS may result in therapeutic treatment and management of diseases such as viral diseases (Ivanov et al., 2016; Ivanov et al., 2017), cancer (Yang et al., 2018), Alzheimer's disease (Huang et al., 2016), diabetes (Maria et al., 2018), atherosclerosis (Georgia et al., 2009) and other life style diseases, like obesity (Masschelin et al., 2020). Because of these relations between viral diseases and ROS, extracts which showed significantly high antiviral activity were selected for further experiments for determining the DPPH and $ABTS^{+}$ assay radical scavenging activity (Table 3.1). Furthermore, the ability of these extracts to suppress the LPS-induced ROS production was also investigated.

3.4.4 DPPH radical-scavenging activity

The principle of the DPPH scavenging activity assay is founded on measuring the colour change of the DPPH radical in the presence of an antioxidant (Figure 3.3). The EC_{50} is defined as the concentration of an antioxidant needed to reduce the absorbance of DPPH by 50 % from the initial absorbance calculated. The goodness-of-fit for the representing the activity on graphs was observed through non-linear regression where the value of the R-squared (R^2) ≥ 0.900 (Appendix, Figure 6.2a). Eight tested extracts demonstrated a noteworthy dose dependent response to DPPH scavenging activity. It was observed that among eight tested extracts, five extracts exhibited EC_{50} values < 10 µg/mL with *A. digitata* (EC_{50} = 4.64 µg/mL)

being the most potent extract, followed by *E. natalensis* (EC_{50} = 5.30 µg/mL), while *S. frutescens* had the least DPPH scavenging activity. These results were comparable to the positive control, ascorbic acid (EC_{50} of 2.50 µg/mL). Irondi et al., (2016) reported that *A. digitata* possess strong DPPH scavenging potential and scavenging concentration (SC_{50} = 0.23 ± 0.01 mg/mL) which is moderate in comparison with the results of this study.

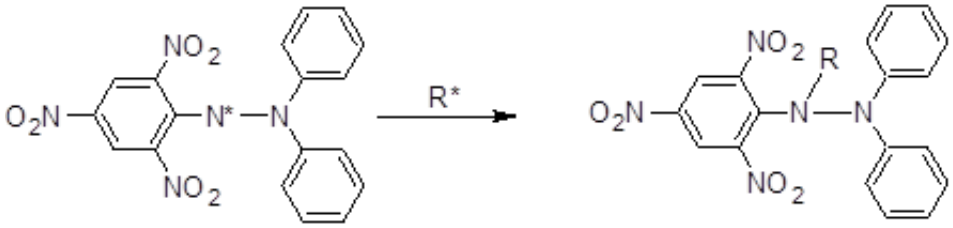

Figure 3.3 Reduction of DPPH scavenged by antioxidants turning the DPPH from purple to a colourless solution (Brand-Williams et al., 1995)

3.4.5 $ABTS^+$ radical scavenging assay

Evaluation of the $ABTS^+$ scavenging activity of eight selected plants, radicals were formulated through the reaction of $ABTS^+$ with potassium per-sulphate ($K_2S_2O_8$) in sterile distilled water. This blue-green $ABTS^+$ solution changes to a light-green colour when exposed to antioxidants capable of donating hydrogen atoms (Figure 3.4) and the reduction may be quantified spectrophotometrically. EC_{50} values were calculated from a scatter plot using a non-linear regression line which showed a good fit coefficient of $R^2 \geq 0.900$ (Appendix, Figure 6.2 B). The results of eight tested extracts demonstrated the reduction of $ABTS^+$ radical and the EC_{50} values were recorded as shown in Table 3.1. Similar to DPPH scavenging results, out of eight tested extracts *E. croceum*, *E. natalensis*, *A. digitata*, in respective order, showed significantly higher $ABTS^+$ reducing power with EC_{50} values < 10 µg/mL, while *H. aureonitens*, *E. transvaalense*, *E. elephantina* and *S. frutescens* having EC_{50} values > 10 µg/mL. It is worth noting that the observed high $ABTS^+$ scavenging activity of the extracts has comparable significant EC_{50} values to positive control, ascorbic acid which exhibited good $ABTS^+$ reducing power with an EC_{50} value of 2.30 µg/mL. Evaluation of DPPH and $ABTS^+$ radical scavenging activities of *E. croceum* acetone leaf extract yielded significant results with IC_{50} of 7.7 µg/mL and 3.1 µg/mL, respectively (Elisha et al., 2016). Odeyemi and Afolayan, (2017) reported the DPPH and $ABTS^+$ scavenging activities of *E. croceum* leaf and bark

acetone extracts. In their study, they observed IC_{50} values 0.09 mg/mL and 0.1 mg/mL for leaf extracts in $ABTS^+$ and DPPH assay, while the bark extract showed activities with IC_{50} of 0.2 and 0.07 mg/mL $ABTS^+$ and DPPH assay, respectively. These results may be credited to the existence of phytochemical classes such as flavonoids, alkaloids and phenolics, which have been reported as bioactive compounds in *E. croceum* (Odeyemi and Afolayan, 2017).

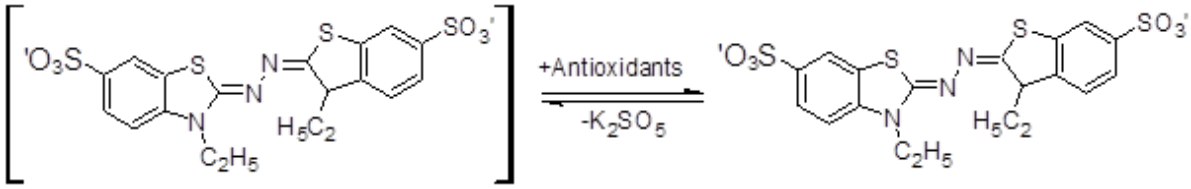

Figure 3.4 Reduction of $ABTS^+$ by hydrogen-donating antioxidants initiating a colour change from blue-green to light green.

3.4.6 Measurement of LPS-induced Intracellular ROS

As plant extracts have shown potent radical scavenging activities, it is worth evaluating the defensive mechanisms of the extracts against oxidative stress. This can be done by assessing the effects of the extracts on the intracellular ROS content in Vero cells by inducing exogenous oxidative stress using lipopolysaccharide (LPS) which is an endotoxin from the Gram-negative bacterium *Escherichia coli*. ROS production was detected using the cell-permeant probe, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), which is a non-fluorescent dye, but it is converted to the fluorescent 2′,7′-dichlorofluorescein (DCF) when acetate groups are cleaved by intracellular esterase (James et al., 2015). The results demonstrate that ROS levels increased considerably to approximately 95 % after exposure to LPS alone, in comparison to the untreated cells. However, pre-treatment with extracts (100 µg/mL) considerably lowered the LPS-induced ROS levels (Figure 3.5). *Adansonia digitata* extract reduced the ROS levels dramatically with 75 % reduction, followed by *E. croceum, E. transvaalense, E. elephantina* and *E. natalensis* with more than 60 % reduction and *A. afra, H. aureonitens* and *S. frutescens* exhibiting less than 50 % reduction. The results obtained in this study correlate well with the exceptional radical scavenging activities.

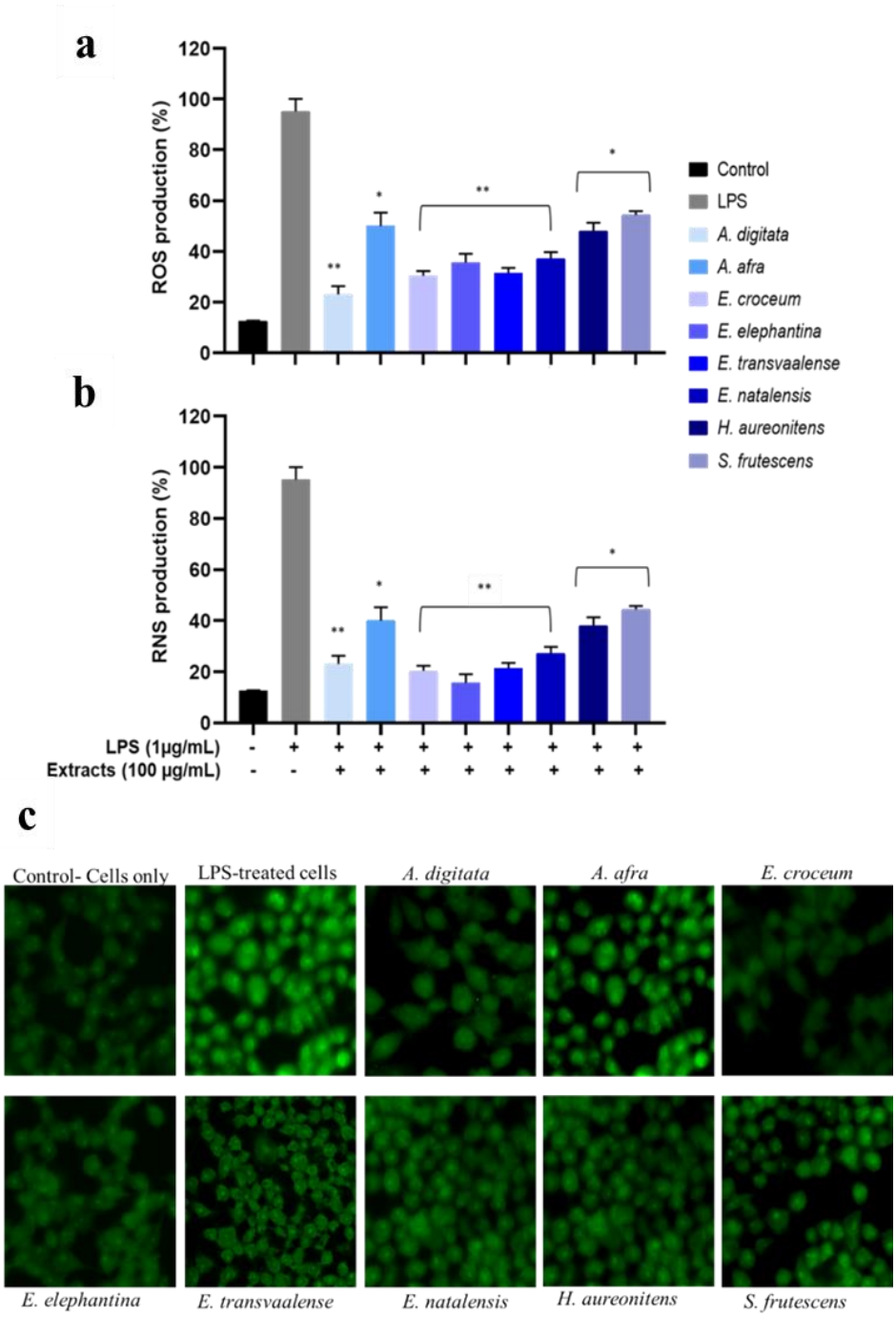

Figure 3.5 Effects of eight extracts with scavenging activity on cell-based ROS (a) and RNS (b) antioxidant. Data analysis was performed using the Duncan multiple range test for means ± SD (*) $p < 0.05$, (**) $p < 0.01$ was measured to designate statistically noteworthy difference. Confocal microscope images indicating the effects of LPS-induced ROS by extracts **(c)**.

3.4.7 Measurement of LPS-induced Intracellular RNS

The RNS experiment was designed to evaluate the inhibition of nitric oxide (NO) production in Vero cells pre-treated with extracts. As shown in Figure 3.5 (b) upon stimulation with LPS (1 µg/mL), an escalated level of NO was produced compared to control cells. Stimulation with extracts (100 µg/mL) considerably lowered the levels of LPS-induced NO. In addition, the immunofluorescence imaging (Figure 3.5 c) revealed that H_2DCF-DA was normally sequestered in the cells and in the nucleus. In this assay the fluorescence intensity can be correlated to production of ROS/RNS. The results showed untreated control cells displayed very low levels of H_2DCF-DA fluorescence intensity signalling low ROS/RNS production. As shown in Figure 3.5 (b), upon stimulation with LPS (1 µg/mL), an increased level of NO was produced when compared to unstimulated cells. Treatment with extracts (100 µg/mL) significantly reduced the levels of LPS-induced NO. Though *E. elephantina* extract showed moderate DPPH and $ABTS^+$ radical scavenging activity, in this case, it exerted the highest suppression activity of NO with inhibition > 80%. Whereas *E. croceum*, *E. transvaalense*, and *E. natalensis* extracts exhibited substantial NO inhibitory activity of approximately 80 %, 78% and 75 %, respectively. Similar to the LPS-induced ROS inhibitory activity, *A. afra*, *H. aureonitens*, and *S. frutescens* showed reasonable attenuation of NO production with a 50 % inhibitory effect. However, ROS/RNS production induced by LPS treatment was significantly elevated as shown by the bright flourescence intensity of the images and extracts reduced the ROS/RNS production shown by dark flourescence intensity of the images. Suppression of ROS/RNS may indicate why the extracts exhibited significantly less toxicity on Vero cells since cellular oxidative stress and cell viability assays can be indicators of elevated ROS production which may result in cell death (Carrasco-Torres et al., 2017).

3.5 Conclusion

Rift Valley Fever is a lethal viral illness and it was registered in the category A-disease that has a potential of rapid and extensive spread by the Office International des Epizooties (OIE). For this reason, more investigation of therapeutic agents is warranted. This study has shown that 50 % aqueous-methanolic medicinal plant extracts can inhibit the RVFV. The *E. croceum* extract significantly decreased the number of $TCID_{50}$ down to 10^2 log of the RVFV infectivity followed by *A. afra* and *A. digitata* with a reduction of up to 10^4 log. An average potency compared to *E. croceum* was observed in *E. transvaalense*, *E. natalensis*, *H. aureonitens* and *S. frutescens*, which lowered the RVFV viral load when 100 µg/mL of the

extracts was added to the virus, which exhibited a $TCID_{50}$ activity range of 10^4–10^5. Furthermore, the real-time monitoring of RVFV infectivity in Vero cells showed inhibitory effects of extracts on RVFV-infected cells. An increase in cell index (CI) values was observed in pre- and post-treatment, with a lag phase from 24 h until 90 h. Moreover, extracts exhibited low cytotoxicity to Vero cells with only *A. afra* and *R. communis* showing LC_{50} values < 200 µg/mL.

The robust scavenging activity presented by the screened extracts necessitate further investigation to better understand their mechanism of action in relation to alleviating oxidative stress induced diseases. Among eight tested extracts against the DPPH radical, five extracts exhibited EC_{50} values < 10 µg/mL with *A. digitata* (EC_{50} = 4.64 µg/mL) and *E. natalensis* (EC_{50} = 5.30 µg/mL) showing the best activity. *Elaeodendron croceum*, *E. natalensis* and *A. digitata* exhibited the best activity (EC_{50} = 4.12, 5.00, 5.04 µg/mL) against the $ABTS^+$ radical, respectively. Furthermore, results from the lipopolysaccharide-induced reactive oxygen species assays demonstrated that plant extracts are potent inhibitors of LPS-induced ROS/RNS in Vero cells. Extracts also showed the reduction in LPS-induced ROS and RNS with high activity > 60 % by *E. croceum*, *E. natalensis* and *A. digitata* in both the ROS and RNS assays. The results obtained in the study correlate well with good radical scavenging activities. This study provides a partial explanation of the relationship between extracts, antiviral, radical scavenging activities and suppression of both ROS/RNS. The elevation of ROS/RNS plays a crucial role in neutralising many viral-induced inflammatory responses. However, ROS/RNS modulate the tolerance of cells to viral replication and regulate host inflammatory and immune responses, thereby resulting in oxidative damage to both the host tissue and progeny virus. Even though there are debates about the role played by elevated ROS/RNS levels in cell signalling, previous research has revealed that amplified levels of ROS/RNS result in a compromised immune system with severe oxidative injuries and viral disease progression. To curb the viral survival, the presence of antioxidants is crucial, as they activate Nrf2 and antioxidant response element (ARE) defence pathways that lead to antioxidant defence system against virus-induced inflammation. In our study, intercellular interaction between the extracts and virus resulted in the reduction in the viral proliferation, possibly by oxidising cell membrane proteins that are responsible for attachment/entry of the virus to mammalian cells. Therefore, bridging this attachment capacity may lead to failure of viral propagation. Moreover, intracellular reduction in ROS/RNS production may lead to the activation of the

antioxidant defensive pathway and suppression of the viral replication by downregulating the nuclear factor-kappaB (NF-κB) pathway that promotes oxidative stress and viral progression. It is envisaged to investigate the performance of these extracts on pro-inflammatory mediators and cytokines such as prostaglandin E2 (PGE2), tumour necrotic factor- α (TNF-α) and NF-kB, to fully understand the signalling pathways during viral infection and post extract treatment. More studies to help comprehend the mechanism of antiviral activity of these extracts such as inhibition of viral replication, viral entry and/or viral protein production should be done. Other studies may include the immunosorbent analyses for the detection of the RVFV antigen.

3.6 References

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Chapter 4

^{1}H-NMR-based metabolomics and UHPLC-qTOF-MS analysis of plants with anti-RVFV activity

4.1 Introduction

Natural products and traditional medicines are of great importance in the field of drug discovery. Natural products offer a diverse variety of secondary metabolites with a range of chemical structures and derivatives that play an important role in chemotherapy (Newman and Cragg, 2006). Drug discovery from natural products has been a slow ongoing process that needs to employ computational technologies that process multidimensional data, coupled with known biological understanding of metabolites and pathways to match the demand of therapeutic agents (Quansah and Karikari, 2016). Investment in technologies such as genomics, transcriptomics, proteomics and more recently, metabolomics can positively influence research output and understanding of therapeutic drug leads in the field of phytomedicine (Okazaki and Saito, 2012). Metabolomics has been widely applied in preclinical studies to determine disease status, diagnosis, food quality and pharmaceutical therapeutics. Therefore, metabolomics in combination with multivariate statistical analysis can be used as a tool for rapid discovery of phytotherapeutics (Gomez-Casati et al., 2013; Choi and Verpoorte, 2014).

The focus of our research was to investigate the use of ^{1}H-NMR-based metabolomics in discriminating selected unrelated medicinal plants and their constituents that are responsible for anti-RVFV activity. The NMR was the preferred analytical instrument for initial data acquisition since it consists of high reproducibility and is a good tool for performing non-targeted analysis as compared to the mass spectrometry (MS) techniques. To assist in the annotation and identification of active metabolites, chemometric tools and databases such as the human metabolome database (HMBD) were used. However, it is generally known that using a single analytical technique is often not sufficient to holistically analyse the diversity of metabolites in a biological sample, therefore, it is important to apply different techniques coupling analytical instruments (Chen et al., 2014); Marshall and Powers, 2018). In this study, to investigate the chemical components of the active samples, UHPLC-qTOF-MS was used for metabolite identification. Although there are scientific reports about the antiviral activities of the selected plants investigated in our study, few compounds have been isolated and identified. Additionally, this is the first study to evaluate the efficacy of selected medicinal plants to inhibit the RVFV.

4.2 Materials and methods

4.2.1 Sample preparation and extraction for ^{1}H-NMR analysis

Pulverised leaves (50 mg) were weighed into a 2 mL Eppendorf tube and directly extracted with 1500 µL deuterated methanol-water (50 %) with 0.1 M monobasic potassium phosphate (KH_2PO_4) buffer in deuterium oxide (D_2O) and 0.05 % trimethylsilypropionic acid sodium salt (TSP). The extraction buffer was adjusted to pH 6.0 using 1N deuterated sodium hydroxide (NaOD). The mixture was homogenised, ultrasonicated for 60 minutes followed by 5 minutes centrifugation at 13000 rpm. The filtration of the mixture was done using a 0.22 µm Millipore syringe filter and the filtrates were transferred to the 5mm NMR tubes and subjected to ^{1}H-NMR analysis. Samples were run in quadruplicate.

4.2.2 ^{1}H-NMR measurement and processing of the spectral data

The ^{1}H-NMR spectra of the samples where generated on a 600 MHz NMR spectroscopy (Varian Inc, California, USA) operating at a frequency of 599.74. The data was manually processed (phase and baseline correction) using MestReNova (version 9.0) software and the TSP used as an internal standard and the chemical shift was referenced to $\delta = 0.0$. The spectral region from $\delta = 0.00$ to $\delta = 9.00$ were bucketed into 0.04 ppm bins and ASCII data file was exported to Excel. The Excel exported ^{1}H-NMR data was analysed by PCA and OPLS-DA on SIMCA-P software (Version 13.0, Umetrics, Umea, Sweden). Data was baseline corrected, normalised and pareto scaled, with regions representing the residual methanol (3.28-3.36 ppm) and water (4.6 -5.0 ppm) ^{1}H-NMR regions removed before further analysis.

4.2.3 Sample preparation for Ultra-High-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-qTOF-MS) analysis

Pulverized leaves (5 mg) were extracted with 1.5 mL of 50 % methanol (LC-grade and ultrapure LC-grade water), homogenized, ultrasonicated for 5 min and the homogenates were centrifuged for 15 min. The filtration of the mixture was done using a 0.22 µm Millipore syringe filter and the filtrates were concentrated by evaporation to dryness. The dried extract was resuspended in 300 µL of 50 % methanol and pipetted into 2 mL HPLC glass vials. Aliquots of extracts were prepared in triplicates and stored at -20 °C prior to analysis.

4.2.4 UPLC-TOF-MS analysis

The chromatographic separation and mass spectrometry detection were performed following a slightly modified method on a UHPLC system with tandem to a SYNAPT G1 Waters HDMS mass spectrometer (Tugizimana et al., 2019; Zhu et al., 2019). A C18 column (150 mm x 2.1 mm, 1.8 µM), thermostatted set at 60 °C was used to obtain the separation of metabolites. Elution solvents (Eluent A: 10 mM formic acid and (Eluent B: acetonitrile with 10 mM formic acid) were injected at 0.4 mL/min. The initial mobile consisted of 98% A and kept for 1 min. The gradient applied started from 98 % A and at 25 min the gradient set to 5% of solvent A. These parameters were kept for 2 min and thereafter returned to initial mobile phase conditions. To avoid variations in data, samples were run in triplicates and solvent blanks were included.

The SYNAPT G1 Waters Q-TOF system was operated in V-optics mode to obtain high resolution mass spectra. Electrospray analysis was done in positive and negative ionization mode for enough detection of compounds. Conditions were set as follows: masses between 1 and 5 mDa were obtained by lock mass using 50 pg/mL leucine enkephalin as reference. The capillary voltage of the instrument was set at 2.5 kV, and 30 V, 4.0 V set for the sampling cone and the extraction cone, respectively. The source temperature of 120 °C and the desolvation temperature was 450 °C. The nebulisation gas (nitrogen gas) was optimised to 550 L h^{-1} and 50 L h^{-1} cone gas was added. The hyphenated system control and data manipulation was determined using the MassLynxv4.1 (SCN 872) and MassFragment v.2.0.w.15 software.

4.3 Results and discussion

4.3.1 ^{1}H-NMR data and multivariate statistical analysis by unsupervised PCA modelling analysis

The methanol-water (50 %) extracts of 20 plants used traditionally as medicine with proven pharmacological antiviral properties were analysed using ^{1}H-NMR spectroscopy, to assess discrimination in polar metabolites according to observed activities on RVFV. Data obtained was subjected to the principal components analysis (PCA), which is an unsupervised, non-biased statistical analysis and outliers which falls outside the 95 % confidence region of the model were identified by a distance to model X (DModX) tool. Identified outliers according to the DModX (Figure 4.1C) were replicates of *Adansonia digitata*, *Artemisia afra*, *Crinum macowanii*, *Euclea natalensis*, *Lobostemon fruticosus*, *Peltophorum africanum*, *Ricinus communis*, *Sutherlandia frutescens*, *Senna petersiana* and *Terminalia sericea*. Variance of 95

% and coefficient R^2 = 0.701 and Q^2 = 0.706 values were used to validate the goodness and predictability of the model. There was no clear clustering or separation among samples, however, the hierarchical cluster analysis (HCA) dendrogram was developed to evaluate whether some groupings from the data can be generated. The HCA dendrogram attempted to group subjects with similar features into three clusters (Figure 4.1A, groupings: red, blue and green circled), which indicate that chemical similarities of the three clusters could be differentiated.

Table 4.1 Samples of the three groups in the PCA plot (Figure 4.1A) as determined by HCA dendrogram

Grouping: Blue circle	**Grouping: Red circle**	**Grouping: Green circle**
Artemisia afra	*Adansonia digitata*	*Carissa edulis*
Helichrysum aureonitens	*Crinum macowanii*	*Elaeodendron transvaalensis*
Senna petersiana	*Euclea natalensis*	*Elaeodendron croceum*
Prunus africana	*Moringa oleifera*	*Elephantorrhiza elephantina*
	Ricinus communis	*Heteropyxis natalensis*
	Sutherlandia frutescens	*Lobostemon fruticosus*
	Ziziphus mucronate	*Peltophorum africanum*

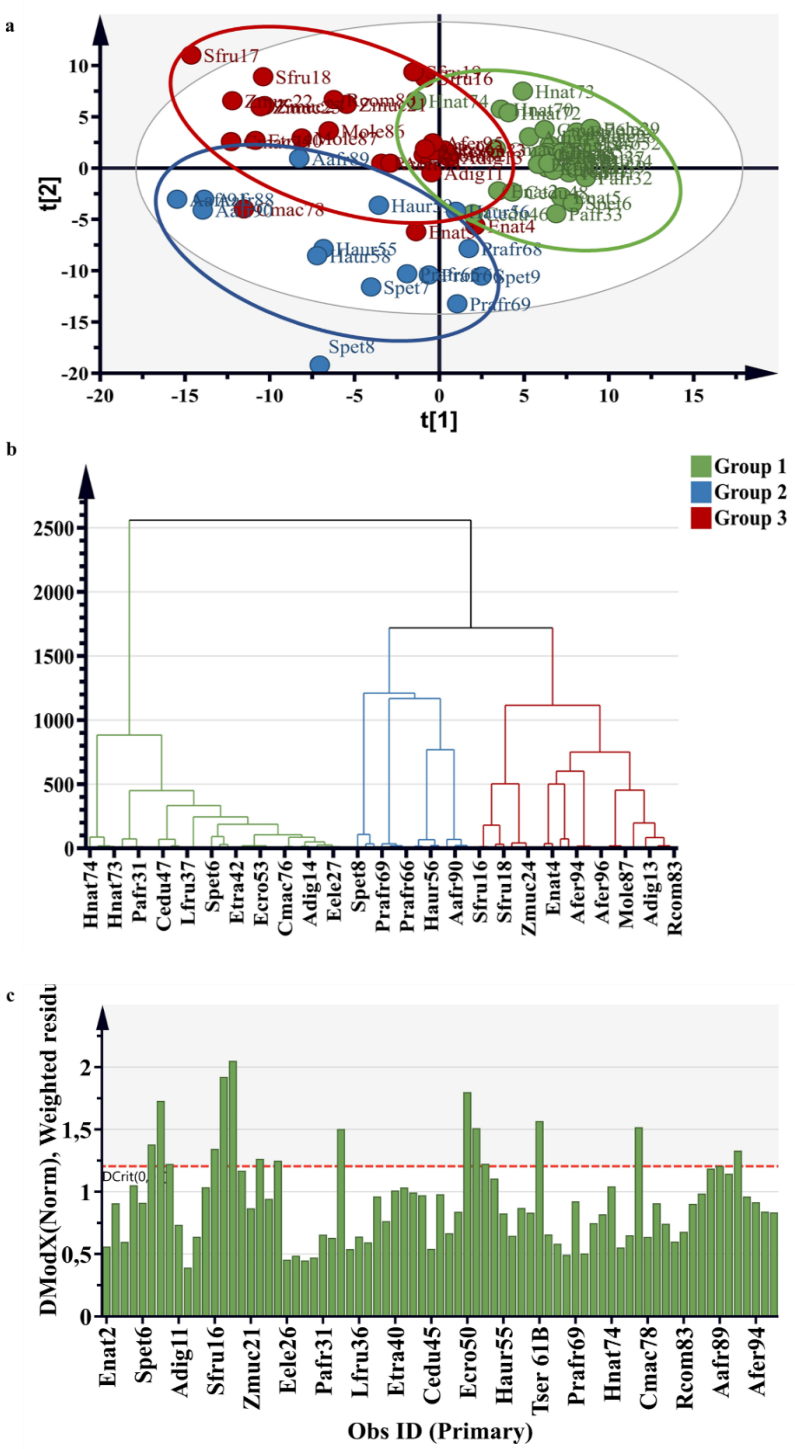


Figure 4.1 Unsupervised PCA plot **(a)** of all the plant extracts. The model explained variation of R^2 = 0.701 and predicted variation of Q^2 = 0.706. The HCA dendrogram **(b)** indicate clear separation of the samples into three groups. This unsupervised model showed an overview of three clusters (groupings: red, blue and green circled) without the knowledge of the bioactivity. Distance to model X (DModX) plot **(c)** showing significant outliers.

4.3.2 Supervised modelling using OPLS-DA and correlation of samples to bioactivity

Correlation of the biological activity of the extracts to responsible variables contributing to their discrimination of the samples, required exposing the ^{1}H-NMR data to the supervised statistical analysis, orthogonal partial least square-discriminant analysis (OPLS-DA). Based on the previous results, the OPLS-DA showed clear separation of the plant extracts into two clusters correlating to the active and non-active samples (Figure 4.2A). The OPLS-DA presents the Hotelling's T2 regions, which defines the 95 % confidence interval of the modelled variation. The correlation coefficient (R^2X= 0.830) showed a goodness of fit of the model and Q^2 = 0.706 indicated the predictive power of the model. It can be inferred that the model is reliable since it is generally known that correlation coefficient (R^2) of the model should be closer to 1, which represents a goodness of fit of a model and $Q^2 > 0.5$ indicating a good prediction of a model.

Table 4.2 Plant extracts clustered into two groups in OPLS-DA plot (Figure 4.2A) with group 1 (red circles) representing the active and group 2 (blue circles) representing less active plants with anti-RVFV activity.

Grouping 1: Red circle	**Grouping 2: Blue circle**
Adansonia digitata	*Aloe ferox*
Artemisia afra	*Carissa edulis*
Euclea natalensis	*Crinum macowanii*
Elaeodendron croceum	*Heteropyxis natalensis*
Elephantorrhiza elephantina	*Lobostemon fruticosus*
Elaeodendron transvaalensis	*Peltophorum africanum*
Helichrysum aureonitens	*Prunus africana*
Sutherlandia frutescens	*Senna petersiana*
	Terminalia sericea
	Ricinus communis
	Ziziphus mucronata

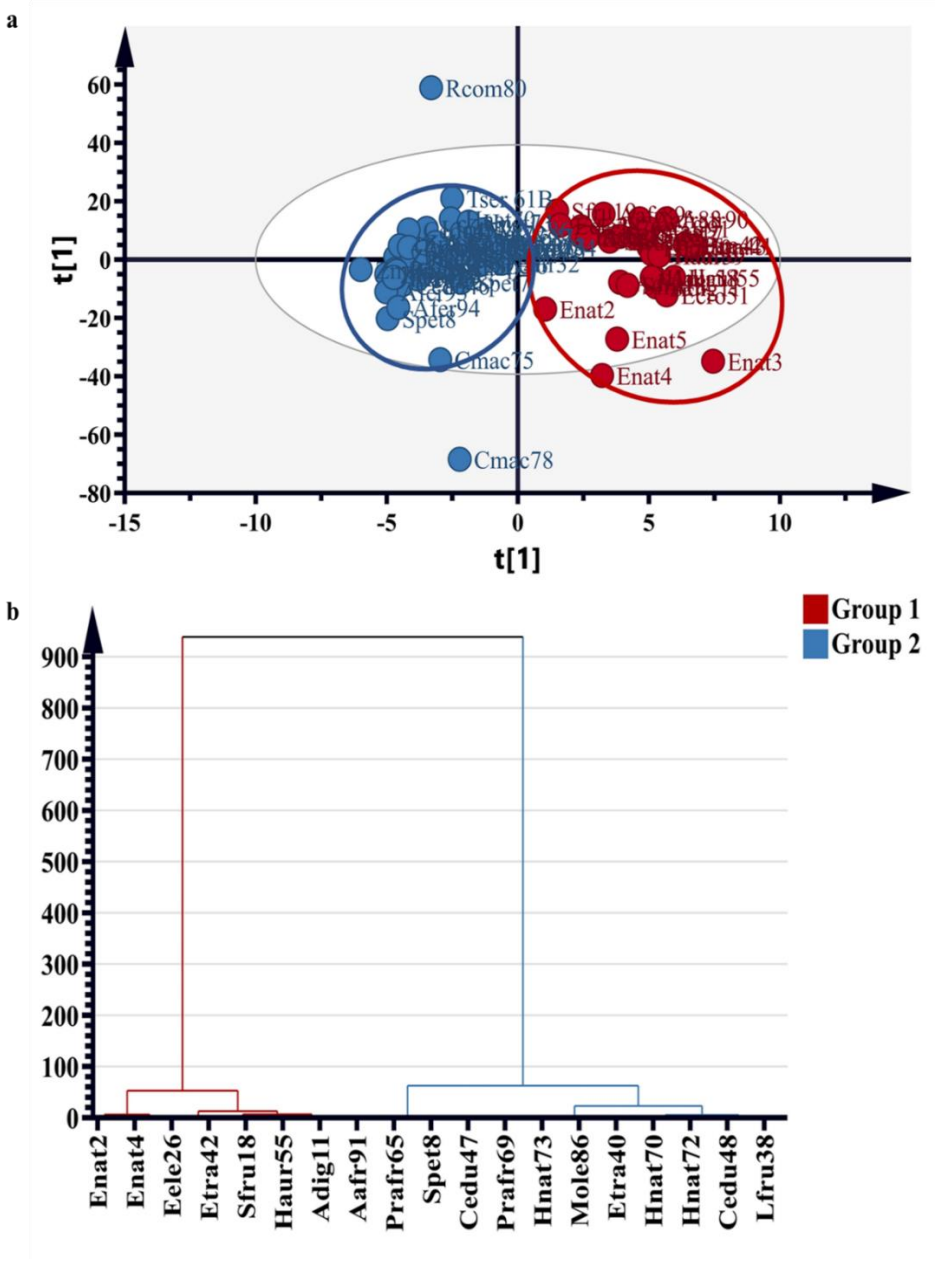


Figure 4.2 Supervised OPLS-DA score plot **(a)** of all the plant extracts. The model explained variation of R^2X= 0.830 and predicted variation of Q^2 = 0.706. HCA dendrogram **(b)** corresponding to clustering in **(a)**. The model shows separation of antiviral active (red circle) and non-active (blue circle) samples.

4.3.3 Statistical validation of the model

The OPLS-DA model was statistically validated using multivariate data analysis and analysed by determining its significance, reliability and predictivity. Validation was performed using the permutation test (n = 100), to evaluate the classification performance, reviver operated characteristic (ROC) which helped to calculate the area under the curve (AUC) and cross-validated predictive residual (CV-ANOVA) where $p < 0.05$ as prescribed by Tugizimana et al., (2016). Permutation analysis was done to further validate the model and 100 permutation tests with a $R^2 = 0.851$ and $Q^2 = 0.561$ (Figure 4.3a) was observed. The ROC (AUC) = 0.9980 with a p-value = $2.30 \times 10^{-12} \pm 2.08$ is presented in Figure 4.3b. Overall, the statistical validation showed reliability and prediction accuracy of the model.

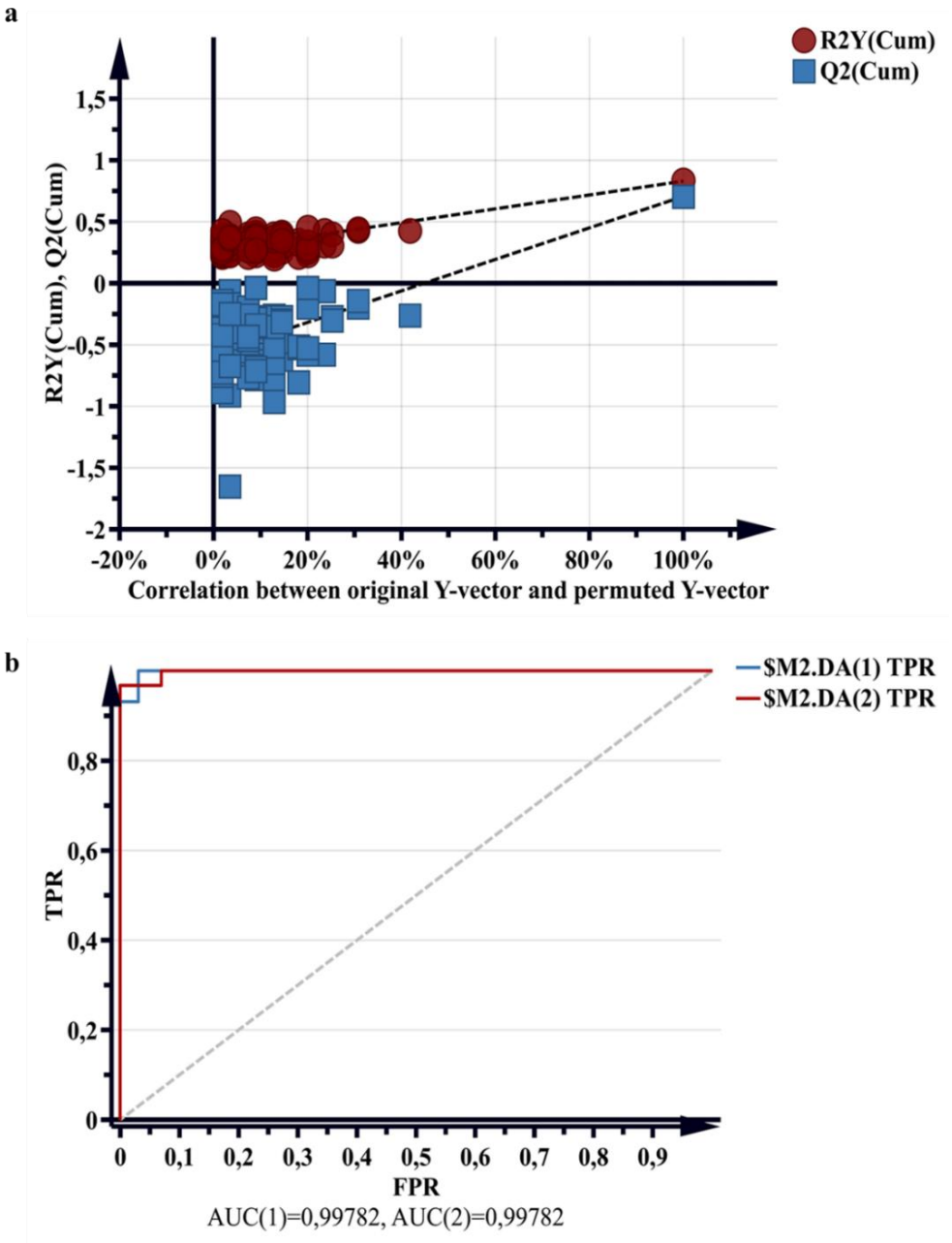


Figure 4.3 Validation models showing results of 100 times permutation test **(a)**, R^2 = 0.851 (green circles), Q2 = 0.561 (blue squares) and ROC (AUC) = 0.9980 **(b)** constructed from the OPLS-DA. These validation plots show that the comparison of predicted groups was accurate.

Discriminative variables were identified using the loading S-plot (Figure 4.4b) which showed bucket values of 2.24, 3.56, 3.68, 4.76, 4.72 and 4.80 ppm as major discriminants of the two groups. The loading S-plots also demonstrated that variables on the two extreme ends of the S-plot are discriminative with variables in group 1 (red circle) of Figure 4.2a being the antiviral active group and the group 2 (blue circle) being the less active. Adopted to the supervised OPLS-DA results, prediction of the variable importance in the projection (VIP) values was performed, which were arranged from the most significant variables from left to right (Figure 4.4a). VIP scores and the S-plot helped to distinguish ^{1}H-NMR regions in order to understand which variables were responsible for the separation and biological activity. Chemical shifts of the VIP scores > 1 were considered significant contributors to the separation of samples.

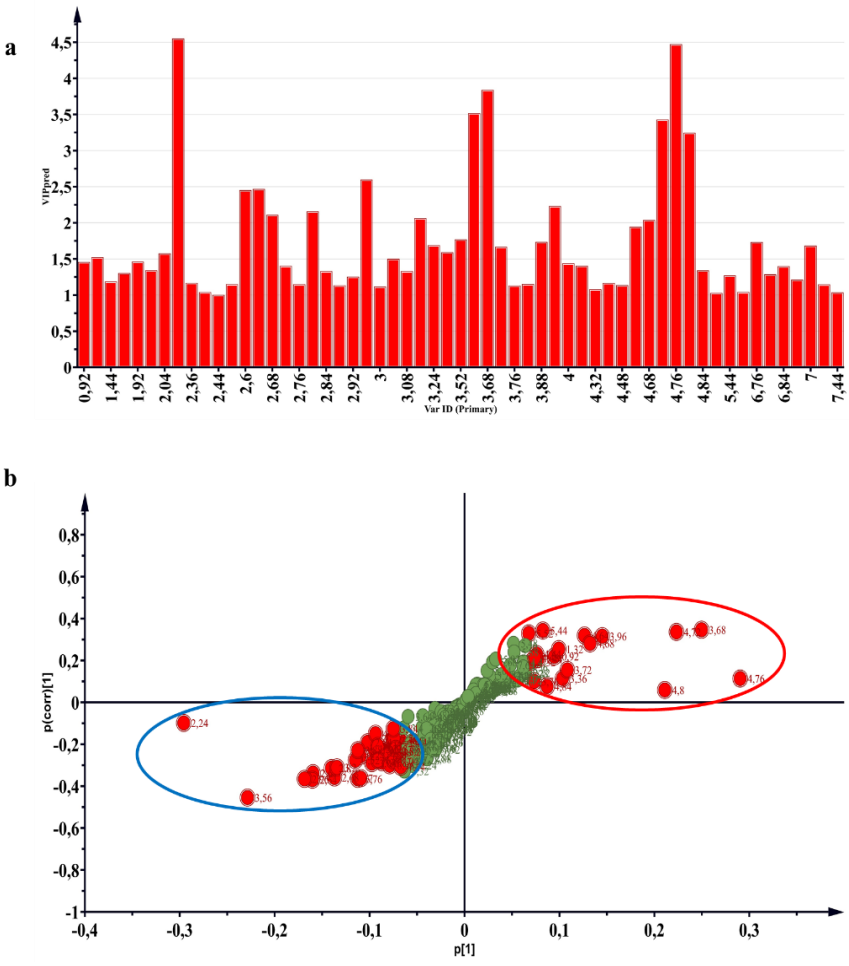


Figure 4.4 VIP score plot **(a)** showing ^{1}H-NMR buckets that contribute to the separation of active and non-active samples with VIP scores greater than 1 which are statistically significant ($p < 0.05$) and loading S-plot **(b)** constructed from the OPLS-DA model showing ^{1}H-NMR regions with variable denoted regions (red circle) contributing to the activity.

In addition, the contribution plot (Figure 4.5) revealed regions that are positively associated to the activity and these regions are 0.92, 1.32, 1.44, 1.68, 1.56, 1.60, 1.64, 2.04, 2.12, 2.20 ppm bucketed values in the aliphatic region. While the presence of some esters and carbonyl compounds were shown by bucket values 3.36, 3.68, 3.72, 3.76, 3.92, 3.96, 4.04, 4.24, 4.32, 4.40, 4.44, 4.48, 4.52, 4.56, 4.64, 4.68, 4.72, 4.76, 4.80, 4.84, 4.88, 5.28, 5.44, 5.48, 5.52, 5.64, 5.68, 5.88, 5.92 and 5.96 ppm in the sugar region were also associated with activity. The presence of aromatic compounds was shown by the bucket values such as 6.0, 6.40, 6.48, 6.92, 7.64 and 7.68 ppm in the aromatic region were thought to contribute positively to activity. However, it is evident that the ^{1}H-NMR spectral regions contributing to the activity of samples are mostly the aliphatic and sugar regions (positive bars). Regions 1.88 – 4.12 ppm (aliphatic) and 4.12 – 5.90 ppm (sugar) showed prominent positively associated peaks, indicating metabolites responsible for activity.

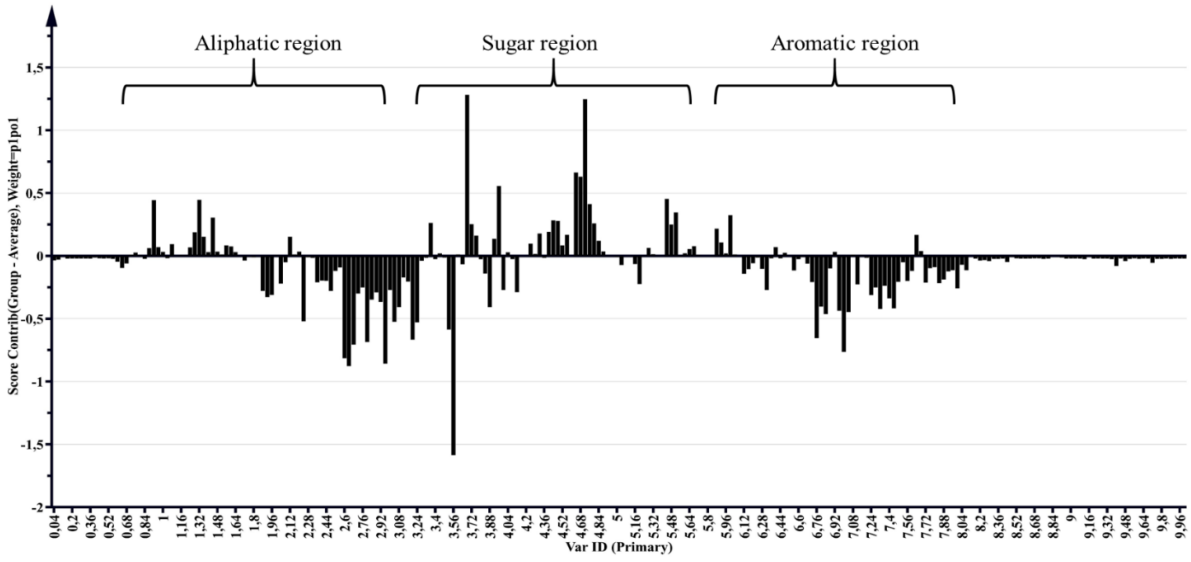


Figure 4.5 OPLS-DA derived contribution plot showing buckets that contribute to activity (positive bars) and non-active (negative bars) samples.

These ^{1}H-NMR regions were compared to diverse classes of secondary metabolites using databases such as HMDB, Chenomx and previous published literature. Metabolites were annotated and their biological significance was successfully elucidated. Highly prominent metabolites which were annotated include amino acids, ferulic acid, trigonelline, chlorogenates and vanillates (Table 4.3).

Table 4.3 Chenomx assisted in annotation of metabolites in anti-RVFV (active) samples. Presented are metabolites, the characteristic peaks' chemical shift (ppm) and peak multiplicity.

Annotated metabolite	Chemical shift (ppm)
Leucine	δ0.96 (d)
Acetate	δ1.98 (s)
Alanine	δ1.50 (d)
Citrate	δ2.50 (d)
Fumarate	δ6.50 (s)
Formate	δ8.47 (s)
Ferulic acid	δ6.38 (d)
Chlorogenic acid	δ7.64 (d)
Vanillate	δ7.44 (dd)
Trigonelline	δ9.15 (s)

The stacked spectra (Figure 4.6) shows common and different metabolite peaks in antiviral extracts. In the high-field region of the ^{1}H-NMR spectra (0.8 – 4.5) of aqueous methanolic extracts, the most abundant peaks correspond to alanine, leucine and acetic acid. Other compounds occurring in this region that are reported in literature include valine, isoleucine, lactate, threonine, arginine, lysine, gamma-aminobutyric acid (GABA), glutamic acid and proline (Lawal et al., 2017). The presence of chlorogenates were detected, such as chlorogenic acid and 4,5-dicaffeolyquinic acid (Liu et al, 2009), as well as epigallocatechin-gallate (Tshikalange et al., 2008; Dhanani et al., 2016) and gallic acid (Dhanani et al., 2016) which have been shown to be more prominent in the aromatic region. In a comprehensive review of compiling the bioactive compounds of *Artemisia* species (Nigam et al., 2019), chlorogenates including crypto-chlorogenic acid, caffeoyl quinic acid and chlorogenic acid were identified, which are difficult to annotate in this study due to the complexity of this region. Analysis of this review further reports on the presence of glycosides of quercetin, catechin and vanillic acid (Nigam et al., 2019) and it seems that a variety of dicaffeoylquinic acid are highly prevalent in *Artemisia* (Liu, Van der Kooy & Verpoorte, 2009) and *Helichrysum* (Heyman et al., 2015) species. Assessment of phytoconstituents from *A. digitata* fruit pulp by LC-MS/q-TOF led to the identification of 46 compounds including protocatechuic acid, caffeic acid, p-hydroxycinnamic acid, p-hydroxybenzoic acid, and chlorogenic acid, to mention a few. The

results warranted further identification of metabolites in active anti-RVFV samples and this confirmation was done using the UHPLC-qTOF-MS.

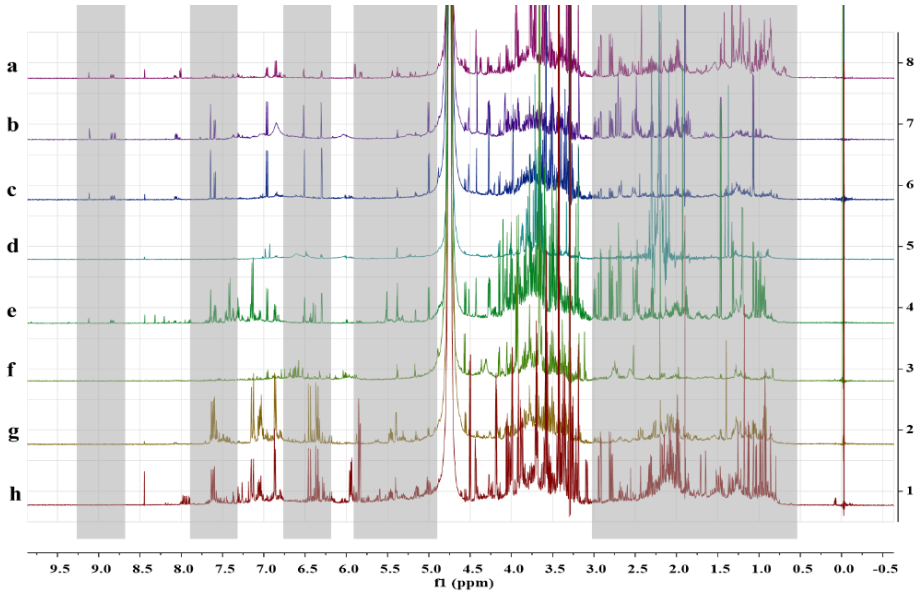


Figure 4.6 Stacked ^{1}H-NMR spectra of eight plant extracts exhibiting anti-RVFV activity. Shaded areas showing similar occurrences of metabolites. **(a)** *Sutherlandia frutescens,* **(b)** *Adansonia digitata,* **(c)** *Elephantorrhiza elephantina,* **(d)** *Euclea natalensis,* **(e)** *Elaeodendron transvaalensis,* **(f)** *Elaeodendron croceum,* **(g)** *Helichrysum aureonitens* **(h)** *Artemisia afra.*

4.3.4 Ultra-High-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-qTOF-MS) metabolite characterization

The eight active antiviral 50 % aqueous methanolic extracts were subjected to UHPLC-qTOF-MS to assess their detailed chemical profiles and the instrument was operated to obtain chromatograms in the positive and negative ion mode which resulted to a total of 61 annotated metabolites. The tentatively identified compounds from the extracts are summarized in Table 4.4 and their chemical structures are presented in Figure 4.7. It is also noteworthy that the UHPLC-qTOF-MS data generally supported the annotation of compounds found in the ^{1}H-NMR metabolomics analysis. The ^{1}H-NMR regions in the contribution plot positively associated with the active samples, aligned very well with the compound peaks for the putatively identified compounds with UHPLC-qTOF-MS, especially in the aliphatic and aromatic ^{1}H-NMR regions,

Data obtained from the UHPLC-qTOF-MS was processed using the MassLynx™ v4.1 SCN 872 software (Waters Corporation, Mildford USA). The mass spectra, retention time (Rt), ion fragments were compared with data from various databases including NIST (National Institute of Standards and Technology) database, DNP (Dictionary of Natural Products: www.dnp.chemnetbase.com), MassBank (USA) and mzCloud (Advanced Mass Spectral Database) to assess the chemical profiles of the extracts. In addition to the in-house data analysis, MAGMa (www.emetabolomics.org) was employed to check for the annotation of the measured masses and mass fragments using KEGG/HMDB/PubChem databases and literature data. Furthermore, the DBE values of annotated compounds were recorded which demonstrates multiple fragmentations in unsaturated compound. DBE values > 1, signifies the presence of several bonds and/or rings in a molecule and the higher values correlated well with conjugated molecules.

The principal classes of compounds characterised in eight antiviral samples include alkaloids, phenolics, flavonoids, terpenoids, steroids, quinones, cardiac glycosides, fatty acids, and amino acids. A group of esterified compounds were prominent in all plant extracts, annotated as chlorogenic acid-type metabolites. The mass spectrum showed peaks at *m/z* 354.09 and *m/z* 516.12 in negative ionisation mode and these masses indicated the presence of caffeoylquinic acids and dicaffeoylquinic acids in *A. afra* and *H. aureonitens* extracts. These compounds include 5-caffeoylquinic acid, 4-caffeoylquinic acid, 3-caffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. Caffeoyl types of compounds are well renowned for their antiviral activity. A study by Urushisaki et al. (2011) found that caffeoylquinic acids such as chlorogenic acid, 3,5-di-O-caffeoylquinic acid (3,5-diCQA), 4,5-di-O-caffeoylquinic acid (4,5-diCQA), 3,4-di-O-caffeoylquinic acid (3,4-diCQA) and 3,4,5- tricaffeoylquinic acid (3,4,5-triCQA), prominent in green propolis plants from Brazil, exhibited potent H1N1 influenza virus inhibition. Another study reported the presence of dicaffeoylquinic and tricaffeoylquinic acids and these compounds were prominent in the anti-HIV potent sample of *Helichrysum populifolium* (Heyman et al., 2015).

Vanillic acid and trigonelline were characterised by peaks at *m/z* 168.04 and 137.04, respectively, with ferulic acid detected in a negative ionisation mode as a product ion at *m/z* 194.05. Previous studies reported the anti-hypoglycaemic, anti-hypolipidemic,

neuroprotective, antimigraine, sedative, antibacterial, antiviral, and anti-tumour properties of trigonelline and ferulic acid (Özçelik et al., 2011; Cui et al., 2013; Song et al., 2016; Mohamadi et al., 2018; Li et al., 2019). Vanillic acid was reported to be the most prominent compound in the root extract of *Rubia cordifolia* which exhibited antiviral activity against rotavirus assessed by the cytopathic reducing effect (CPE) of the virus on MA-104 cells (Sun et al., 2016).

Elemental composition of carboxylated cyclohexanepolyol was shown existed as product ion at *m/z* 191.05 and were assigned to quinic acid. Quinic acid, chlorogenic acid and caffeic acid exhibit HIV integrase and HIV replication inhibitory effects (Kwon et al., 2000). Moreover, the quinic acid derivatives 3,5-di-O-caffeoylquinic acid and 3,4-di-O-caffeoylquinic acid inhibited the respiratory syncytial virus (RSV) with IC_{50} values of 2.33 and 1.16 µM, respectively (Li, But and Ooi, 2005). Furthermore, a study investigating the ability of caffeic acid, quinic acid, and chlorogenic acid from the crude coffee extracts against hepatitis-B virus revealed inhibitory potency both intracellular and intracellular. In intercellular experiments caffeic acid, quinic acid and chlorogenic acid, had IC_{50} values of 1.3, 1.6 and 0.7 µM, respectively. On the other hand, caffeic acid, quinic acid, and chlorogenic acid, possessed IC_{50} values of 3.9, 10.1, and 1.2 µM when tested extracellularly (Wang et al., 2009).

Two hydroxylated fatty acid derivatives of linoleic acid were identified by peaks at *m/z* 294.22 and *m/z* 296.24, and were annotated for the first time in all the antiviral active samples and are reported as 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid and 13-hydroxy-9Z,11E-octadecadienoic acid. The presence of fatty acids was also confirmed with the strong positive association of the aliphatic regions in the ^{1}H-NMR metabolomics analysis. The antiviral activity of various naturally occurring fatty acids has been demonstrated. Kaigongi et al. (2020) documented for the first time the presence of the unsaturated fatty acid, 17-hydroxylinolenic acid from a multi-purpose medicinal plant *Dodonaea viscosa* Jacq (Sapindaceae) with significant pharmacological activities ranging from antimicrobial, anti-inflammatory, anti-allergic and anticancer activities (Mundt et al., 2003; Korinek et al. 2017). Fatty acids, particularly the medium chain saturated and long chain unsaturated fatty acids were reported to reduce viral concentrations of vesiculovirus (VSV) and herpes simplex virus (HSV) in cell cultures by 10,000 fold (Aldridge, 2020). Another oxygenated fatty acid, 10S, 17S-dihydroxydocosahexaenoic acid, also known as protectin-D1 (PD1) has been shown to inhibit

the H5N1 influenza virus replication by interfering with the virus RNA nuclear export. Protectin-D1 (PD1) exhibited a 30 % reduction of the viral load with a $TCID_{50} = 1x10^5$ (Morita et al., 2013). The antiviral assessment of *Phyllocaulis boraceiensis* mucus and its fractions against influenza A strain using Madin–Darby canine kidney (MDCK) cells revealed that the mucus and fractions reduced the viral-induced cytopathic effects by more than 80% and inhibits viral replication. HPLC-DAD-ESI-MS/MS analysis of *Phyllocaulis boraceiensis* mucus and its fractions showed the presence of hydroxy polyunsaturated fatty acids as major antiviral constituents. This deduce that the hydroxy polyunsaturated fatty acids obtained interferes with the binding ability of the virus to host cell receptors thereby decreasing the viral load (Rita et al., 2018).

The application of HODE compound (5 - 100 µM) reduced the cell viability of the breast cancer cell line, MCF-7 and MDA to approximately 53.25% and 62.5%, respectively (Tavakoli-Yaraki and Karami-Tehrani, 2013). Another study on the anticancer activity of coriolic acid (13-(S)-hydroxy-9Z,11E-octadecadienoic acid) isolated from *Salicornia herbacea* was reported to suppress breast cancer stem cells by c-Myc proteins modulation. Coriolic acid exhibited IC_{50} values of 289.3 and 386.9 µM against MDA and MCF-7 cells, respectively (Ko et al., 2020). Lines and Lines, (2015) demonstrated that MCF-7 cells treated with 100 µM of 13(S)-HODE exhibited about 53.25% and 62.5% cell viability reduction after 48 and 72 h, respectively. Furthermore about 57.75% and 62.25% cell viability reduction was observed when the MDA cells was treated with 100 µM of 13(S)-HODE for 48 and 72 hr, respectively. However, the transcriptional protein targeted by the 13(S)-HODE was the peroxisome proliferator-activated receptors-δ (PPAR-δ) mRNA expression. The findings in the latter study are in agreement with a study where an antineoplastic activity of 15-(S)-hydroxyeicosatetraenoic acid (15S-HETE) and 13(S)-hydroxy-9Z,11E-octadecadienoic acid (13S-HODE) were investigated on the lung cancer cells. 15(S)-HETE and 13(S)-HODE significantly reduced the cell viability of lung cancer cells showing IC_{50} values of approximately 40 µM and this was through the activation of the PPAR ligands (Li et al., 2015). A fatty acid methyl ester [(9Z,11E)-13hydroxy-9,11-octadecadienoic acid and (9Z,llE)13-oxo-9,11-octadecadienoic acid] isolated from leaves and twigs of *Ehretia dicksonii* possessed anti-inflammatory activity on mouse ears and lipoxygenase inhibitory activities of 63% and 79% was observed, respectively (Dong et al., 2000).

A flavonoid, kaempferol **(14)** and flavonol glycoside, kaempferol 3-O-rutinoside **(15)** were also tentatively identified at *m/z* 286.04 and *m/z* 594.15, respectively. Another review reported flavonoids as Enterovirus A71 (EV-A71) inhibitors. This review showed that kaempferol with an IC_{50} value of 52.75 μM inhibits the EV-A71 sub-genotype C4 strain and decreased the viral RNA copies and protein synthesis (Lalani and Poh, 2020). A bioflavonoid rutin **(19)**, also known as quercetin-3-rutinoside was detected by a peak at *m/z* 610.15. According to our analysis rutin was found in most tested samples, but not in *E. natalensis* and *E. croceum* extracts. Ganeshpurkar and Saluja, (2017) documented a review on the pharmacological potential of rutin and they showed that rutin exhibits a range of biological activities including anticancer, analgesic, antiarthritic and antiviral properties. Rutin with an IC_{50} value of 110 μM inhibited the replication stage of infection (Lalani & Poh, 2020), however, it could not inhibit the viral replication of Dengue virus type-2 (DENV-2) (Keivan et al., 2014).

Table 4.4 List of compounds annotated from eight anti-RVFV leaf extracts analysed by UHPLC-qTOF-MS showing retention times (Rt), mass-to-charge ratio (*m/z*), molecular formula, double-bond equivalent (DBE 0–21), proposed metabolite and mode of detection. The table also shows in which plants were annotated metabolites present: *Artemisia afra* (Aa), *Adansonia digitata* (Ad) *Euclea natalensis* (En), *Elaeodendron croceum* (Ec), *Elaeodendron transvaalensis* (Et), *Elephantorrhiza elephantina* (Ee), *Helichrysum aureonitens* (Ha), *Sutherlandia frutescens* (Sf).

Rt (min)	Observed mass (*m/z*)	Calculated mass (*m/z*)	Fragment Ions	DBE count	Molecular Formula	Annotated Metabolites	Comments	Mode of detection	Plant species
0.93	138.0555	137.0477	110.06; 94.06	5	$C_7H_7NO_2$	Trigonelline	MassBank (USA)	Positive mode	Ad, Ee, Sf
1.14	193.0488	354.0951	-	8	$C_{16}H_{18}O_9$	Chlorogenic acid	Product ion; trace level	Negative mode. Ferulic fragment product (of chlorogenic acid)	Ad
2.98	353.0872	354.09508	191.1; 179.0	8	$C_{16}H_{18}O_9$	3-Caffeoylquinic acid	(Clifford et al., 2008)	Negative mode	Aa, Ha
4.53	353.0872	354.09508	191.1; 179.1 (low intensity)	8	$C_{16}H_{18}O_9$	5-Caffeoylquinic acid	(Clifford et al., 2008)	Negative mode	Aa, Et, Ha
4.69	353.0842	354.09508	173.0; 179.0; 191.1	8	$C_{16}H_{18}O_9$	4-Caffeoylquinic acid	(Clifford et al., 2008)	Negative mode	Aa, Ha
6.03	515.1157	516.12678	353.1; 191.1; 335.1	14	$C_{25}H_{24}O_{12}$	4,5-Dicaffeoylquinic acid	(Clifford et al., 2008)	Negative mode	Aa, Ha
8.87	515.1195	516.12678	173.0; 335.1	14	$C_{25}H_{24}O_{12}$	3,4-Dicaffeoylquinic acid	(Clifford et al., 2008)	Negative mode	Aa, Ha
9.19	515.1196	516.12678	353.1; 191.1	14	$C_{25}H_{24}O_{12}$	3,5-Dicaffeoylquinic acid	(Clifford et al., 2008).	Negative mode	Aa, Ha

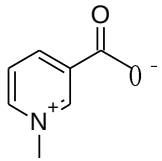
1.14	193.0488	194.0579	-	6	$C_{10}H_{10}O_4$	Ferulic acid	NIST 2014; product ion	Negative mode. Ferulic fragment product (of chlorogenic acid)	Aa, Ha
1.14	167.0325	168.0423	-	5	$C_8H_8O_4$	Vanillic acid	Product ion	Negative mode	Et
2.50	315.1063	316.1158	153.05	5	$C_{14}H_{20}O_8$	Hydroxytyrosol glucoside/` vanilloloside	Mass Fragment	Negative mode	Ee
2.60	153.0546	152.0473	-	5	$C_8H_8O_3$	4-Hydroxyphenylacetate/ vanillin	Product ion	Positive mode	Et
1.34	191.0546	192.0634	173.0; 128.0; 111.0	2	$C_7H_{12}O_6$	Quinic acid	MAGMa, KEGG/HMDB /PubChem	Negative mode	Aa, Ad, Et, Ha
	294.2279	293.2079	-		$C_{18}H_{30}O_3$	13S-Hydroxy-9Z,11E,15Z-octadecatrienoic acid	MAGMa, KEGG/HMDB /PubChem	Negative mode	Aa, Ad, En, Ec, Et, Ee, Ha, Sf
	296.2436	295.2136	-		$C_{18}H_{32}O_3$	13-Hydroxy-9Z,11E-octadecadienoic acid	MAGMa, KEGG/HMDB /PubChem	Negative mode.	Aa, Ad, En, Ec, Et, Ee, Ha, Sf
2.12	166.0833	165.0790	120.1	5	$C_9H_{11}NO_2$	Phenylalanine	NIST 2014	Positive mode	Aa, Ad, Ee, Ha, Sf
3.27	205.0968	204.0899	188.1; 159.1; 146.1	7	$C_{11}H_{12}N_2O_2$	L- Tryptophan	NIST 2014	Positive mode	Aa, Ad, Ee, Ha, Sf
12.23	301.0305	302.0427	273.04; 178.99; 151.00	11	$C_{15}H_{10}O_7$	Quercetin	NIST 2014 & User Library	Negative mode	Ee

5.12	771.1982	772.2062	609.1; 462.1; 301.0	14	$C_{33}H_{40}O_{21}$	Quercetin 3-rutinoside-7-glucoside	MAGMa, KEGG/HMDB /PubChem	Negative mode	Ad
8.24	609.1454	610.1534	300.02	13	$C_{27}H_{30}O_{16}$	Rutin	NIST 2014	Negative mode	Aa, Ad, Ec, Et, Ee, Ha, Sf
8.24	609.1438	610.1534	300.02	13	$C_{27}H_{30}O_{16}$	Rutin hydrate	Reference standard; NIST 2014	Negative mode	Aa, Et, Ha
9.33	593.1509	594.1585	285.0	13	$C_{27}H_{30}O_{15}$	Kaempferol 3-O-rutinoside	MAGMa, KEGG/HMDB /PubChem	Negative mode	Aa, Ad, Et
14.18	285.0374	286.0477	-	11	$C_{15}H_{10}O_6$	Kaempferol	NIST 2014	Negative mode	Aa, Ad, En, Et, Ee
3.76	307.0763	306.0740	289.07; 181.05; 139.04	8	$C_{15}H_{14}O_7$	Epigallocatechin	NIST 2014; MassFragment	Positive mode	Ec
4.15	289.0716	290.0790	245.08; 205.05; 179.03	9	$C_{15}H_{14}O_6$	Catechin	NIST 2014 & mzCloud	Negative mode	En, Ee
4.23	289.0692	290.0790	245.08; 205.05; 179.03	9	$C_{15}H_{14}O_6$	Epi-catechin	NIST 2014 & mzCloud	Negative mode	En, Ee
4.31	319.0769	320.0896	289.07	9	$C_{16}H_{16}O_7$	4'-O-Methyl-(-)-epigallocatechin	(Yelani et al., 2010); Yelani and Meyer 2009	Negative mode	En
8.24	463.0845	464.0955	316.02	12	$C_{21}H_{20}O_{12}$	Myricitrin	NIST 2014	Negative mode	En
6.06	591.1492	592.1581	-	18	$C_{31}H_{28}O_{12}$	Proanthocyanidin A	DNP & KnapSack	Negative mode	Ec
10.37	435.1311	436.1370	273.08; 167.03; 125.04	10	$C_{21}H_{24}O_{10}$	Phlorizin	NIST 2014	Negative mode	Ec

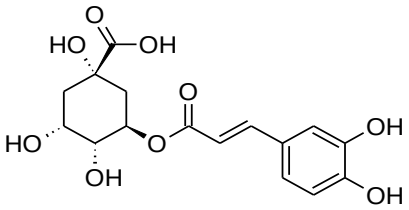
11.20	317.0673	316.0583	302.04; 285.04; 153.02	11	$C_{16}H_{12}O_7$	Isorhamnetin	NIST 2014	Negative mode	En
13.52	271.0608	272.0685	151.00	10	$C_{15}H_{12}O_5$	Naringenin	Yelani et al. 2010;Yelani and Meyer 2009 NIST 2014 User Lib	Negative mode	En, Ec
6.04	319.0833	320.0896	-	9	$C_{16}H_{16}O_7$	Ourateacatechin/ 4-methyl-epigallocatechin	KnapSack	Negative mode	Ec
14.11	285.0400	286.0477	-	11	$C_{15}H_{10}O_6$	Luteolin	MAGMa, KEGG/HMDB /PubChem	Negative mode	Et
7.61	609.1436	610.1534	447.09; 285.04	13	$C_{27}H_{30}O_{16}$	Luteolin diglycoside	MAGMa, KEGG/HMDB /PubChem	Negative mode	Et
10.40	447.0905	448.1006	285.04	12	$C_{21}H_{20}O_{11}$	Luteolin glycoside	NIST 2014;	Negative mode	Et
3.43	577.1331	578.1424	451.10; 425.08; 407.07; 289.07	18	$C_{30}H_{26}O_{12}$	Procyanidin B2/B5	NIST 2014 & MassBank (USA)	Negative mode	Ee
9.31	593.1516	594.1585	285.04	13	$C_{27}H_{30}O_{15}$	Nicotiflorin/ kaempferol-glucoside-rhamnoside	NIST 2014 & User Library	Negative mode	Aa, Ee, Ec
24.90	429.3640	428.3654	-	5	$C_{29}H_{48}O_2$	Elaeodendrol	Product ion	Positive mode	Et
16.27	651.4096	652.41865	-	7	$C_{36}H_{60}O_{10}$	Sutherlandioside A	Avula 2014; DNP	Negative mode	Sf
15.35	651.4105	652.41865	-	7	$C_{36}H_{60}O_{10}$	Sutherlandioside B	Avula 2014; DNP	Negative mode	Sf
16.39	649.3954	650.40300	-	8	$C_{36}H_{58}O_{10}$	Sutherlandioside C	Avula 2014; DNP	Negative mode	Sf
16.42	633.4003	634.40808	-	8	$C_{36}H_{58}O_9$	Sutherlandioside D	Avula 2014); DNP	Negative mode	Sf

10.78	539.1742	540.1843	377.12; 345.09; 307.08; 275.09	10	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside	NIST 2014; MassFragment	Negative mode	Et
9.43	701.2298	702.2371	539.19; 377.12; 307.08; 275.08	11	$C_{31}H_{42}O_{18}$	Oleuropeinyl monoglucoside	MAGMa, KEGG/HMDB /PubChem	Negative mode	Et
11.51	539.1710	540.1843	377.12; 345.09; 307.08; 275.09	10	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside	NIST 2014; MassFragment	Negative mode	Et
4.37	603.2842	602.2727	471.24; 441.23; 309.18	12	$C_{32}H_{42}O_{11}$	Plantagiolide	DNP	positive mode	Ec
12.42	535.2904	536.2985	373.24; 161.04	8	$C_{29}H_{44}O_{9}$	Digitoxigenin glucoside	Prinsloo and Meyer 2007	Negative mode	Ec
12.16	533.2751	532.2672	515.26; 387321; 369.20; 351.19	10	$C_{29}H_{40}O_{9}$	Corotoxigenin-rhamnopyroside	DNP & KnapSack	Negative mode	Ec
16.10	377.0972	376.0947	359.09; 345.11	15	$C_{22}H_{16}O_{6}$	Natalenone/ naphthoherniarin	DNP	Positive mode	En
8.27	739.1750	740.17999	637.15; 595.13; 300.03	15	$C_{32}H_{36}O_{20}$	Sutherlandin A	Avula 2014; DNP	Negative mode	Sf
8.60	739.1724	740.17999	637.15; 595.13; 300.03	15	$C_{32}H_{36}O_{20}$	Sutherlandin B	Avula 2014; DNP	Negative mode	Sf
9.23	723.1805	724.18508	621.15; 579.13; 284.03	15	$C_{32}H_{36}O_{19}$	Sutherlandin C	Avula 2014; DNP	Negative mode	Sf
9.46	723.1805	724.18508	621.15; 579.13; 284.03	15	$C_{32}H_{36}O_{19}$	Sutherlandin D	Avula 2014; DNP	Negative mode	Sf
8.27	739.1750	740.17999	637.15; 595.13; 300.03	15	$C_{32}H_{36}O_{20}$	Sutherlandin A	Avula 2014; DNP	Negative mode	Sf
6.27	431.1926		-	5	$C_{19}H_{30}O_{8}$	Roseoside	FA adduct	Negative mode	En
7.34	625.1299		479.08; 463.08; 316.02	13	$C_{27}H_{30}O_{17}$	Myricetin-3-neohesperidoside	MAGMa, KEGG/HMDB /PubChem	Negative mode	En

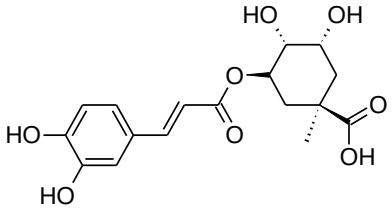
9.63	477.0996		447.09; 331.05; 316.02	21	$C_{22}H_{22}O_{12}$	Estragonoside	MAGMa, KEGG/HMDB /PubChem	Negative mode	En
9.69	447.0910		301.04	12	$C_{21}H_{20}O_{11}$	Quercitrin	MAGMa, KEGG/HMDB /PubChem	Negative mode	En
10.16	965.2995		671.20	9	$C_{37}H_{58}O_{29}$	Lipid	MAGMa, KEGG/HMDB /PubChem	Negative mode	En
11.12	677.4996		461.11	6	$C_{40}H_{70}O_{8}$	Lipid	MAGMa, KEGG/HMDB /PubChem	Negative mode	En



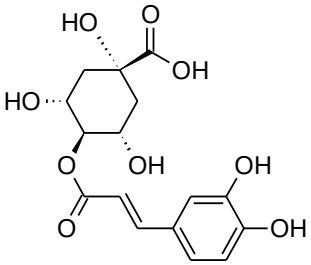
[1]: Trigonelline



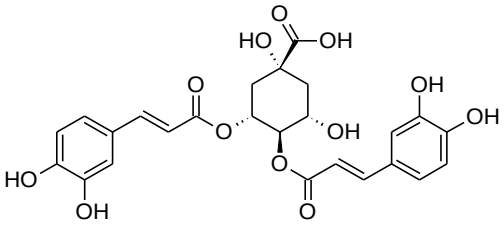
[2]: Chlorogenic Acid (3-Caffeoylquinic acid)



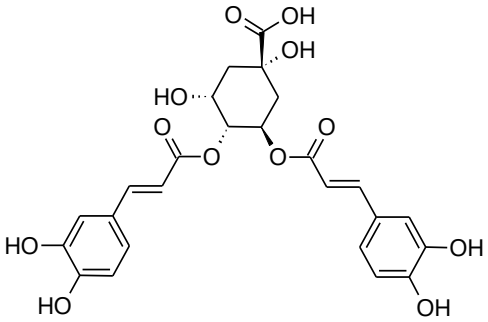
[3]: 5-Caffeoylquinic acid



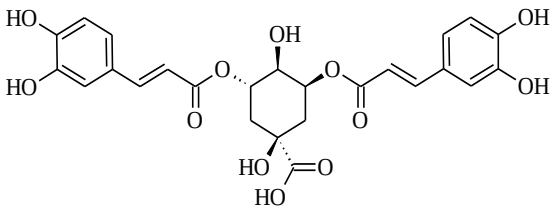
[4]: 4-Caffeoylquinic acid



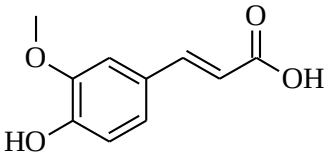
[5]: 4,5-Dicaffeoylquinic acid



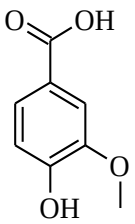
[6]: 3,4-Dicaffeoylquinic acid



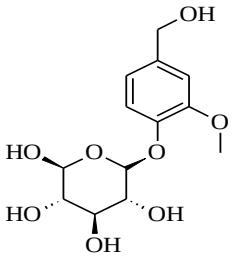
[7]: 3,5-Dicaffeoylquinic acid



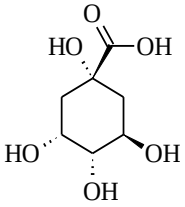
[8]: Ferulic Acid



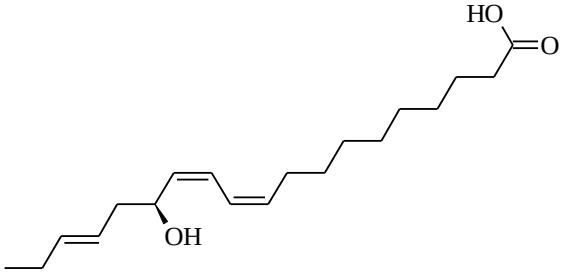
[9]: Vanillic acid



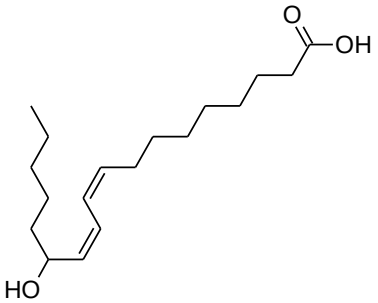
[10]: Vanilloloside



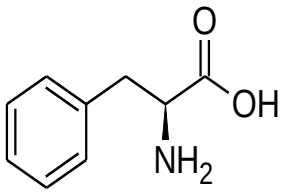
[11]: Quinic acid



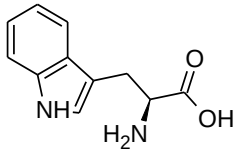
[12]: 13S-Hydroxy-9Z,11E,15Z-octadecatrienoic acid



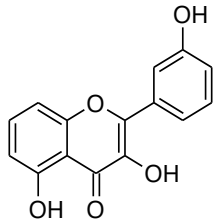
[13]: 13-Hydroxy-9Z,11E-octadecad-ienoic acid



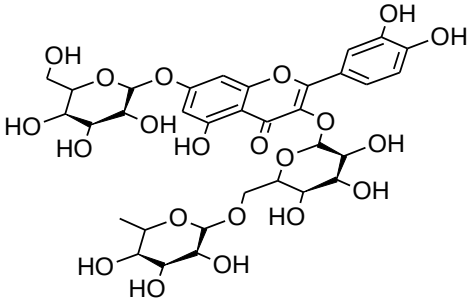
[14]: Phenylalanine



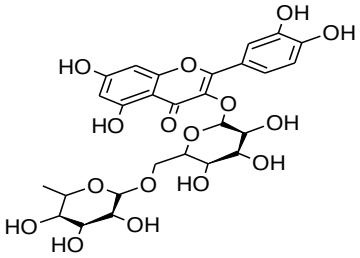
[15]: Tryptophan



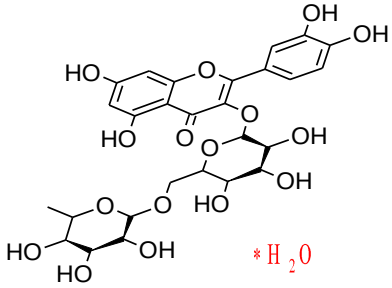
[16]: Quercetin



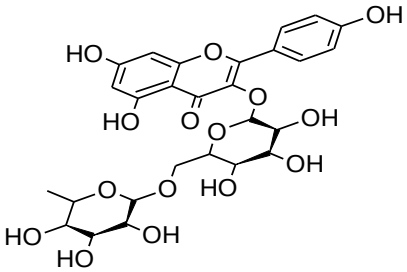
[17]: Quercetin 3-rutinoside-7-glucoside



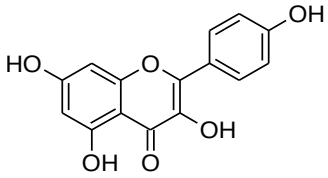
[18]: Rutin



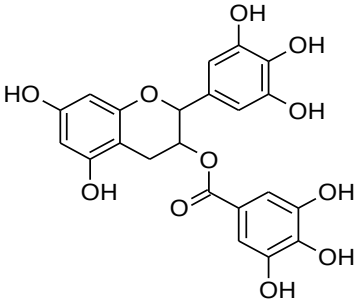
[19]: Rutin hydrate



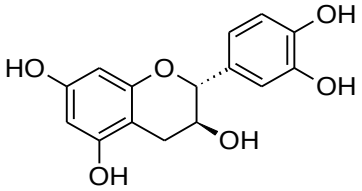
[20]: Kaempferol 3-O-rutinoside



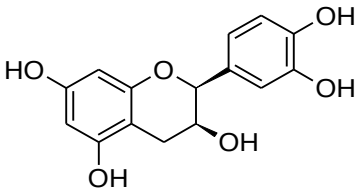
[21]: Kaempferol



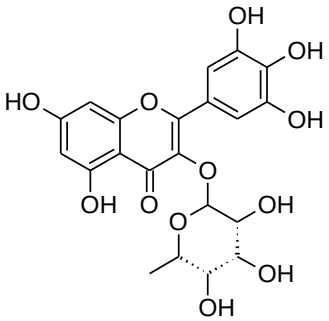
[22]: Epigallocatechin gallate



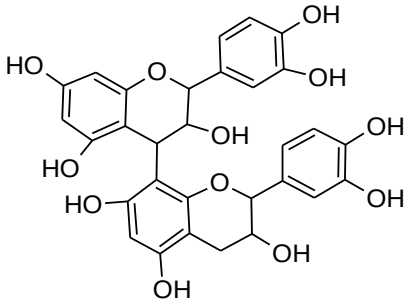
[23]: Catechin



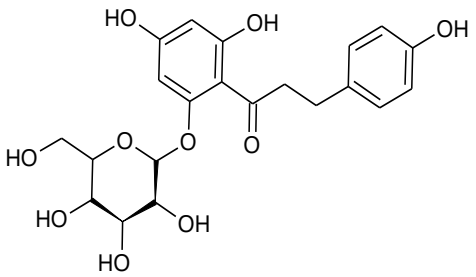
[24]: Epicatechin



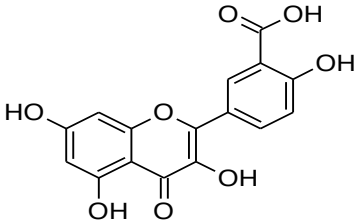
[25]: Myricitrin



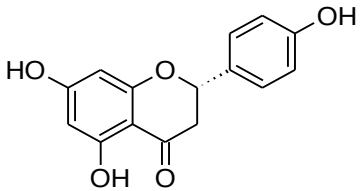
[26]: Proanthocyanidin A



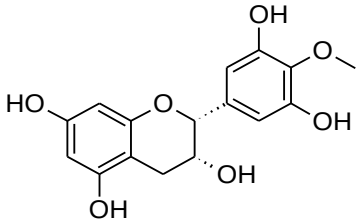
[27]: Phlorizin



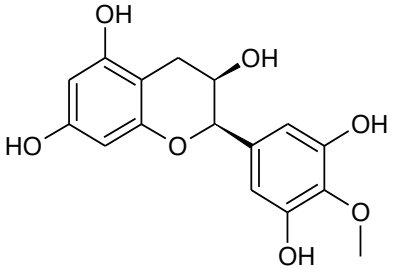
[28]: Isorhamnetin



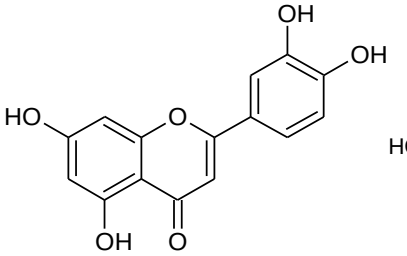
[29]: Naringenin



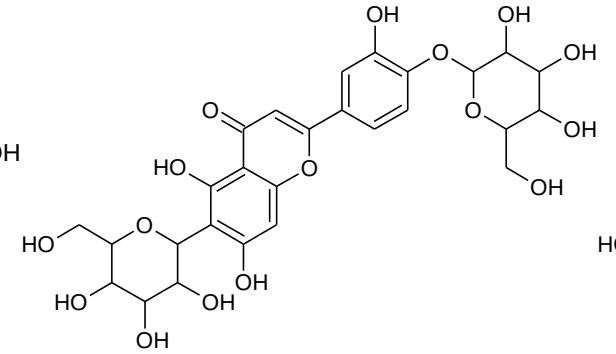
[30]: Ourateacaechin



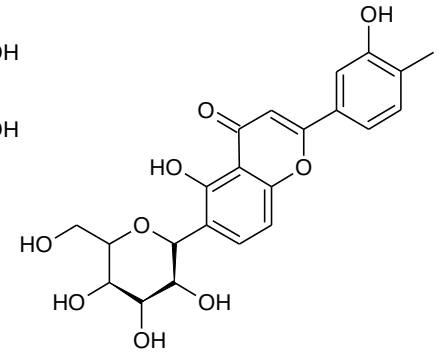
[31]: 4-Methyl-epigallocatechin



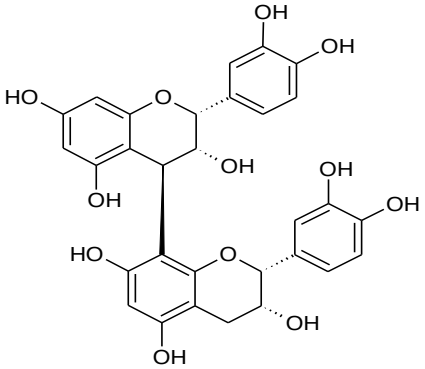
[32]: Luteolin



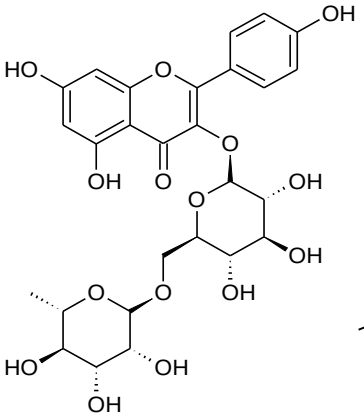
[33]: Luteolin diglycoside



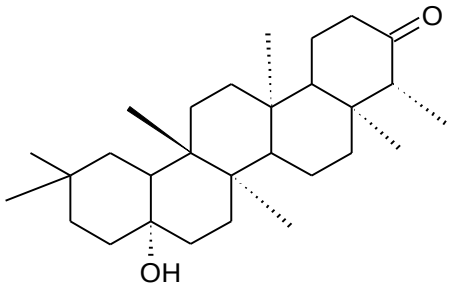
[34]: Luteolin glycoside



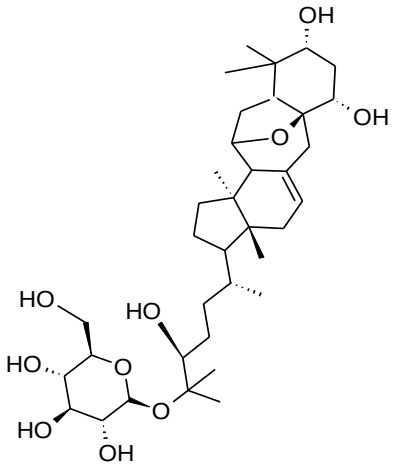
[35]: Procyanidin



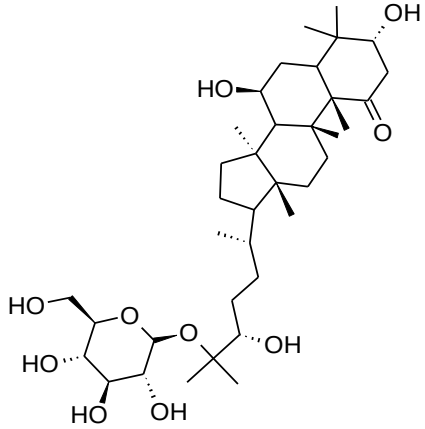
[36]: Nicotiflorin



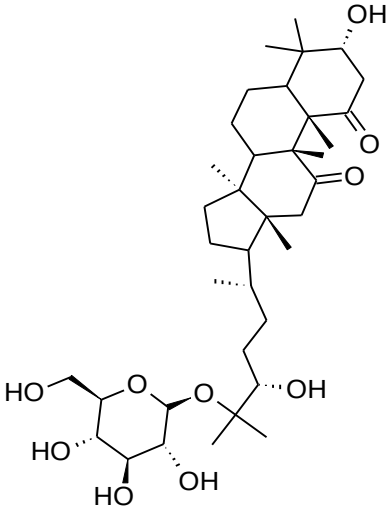
[37]: Elaeodendrol



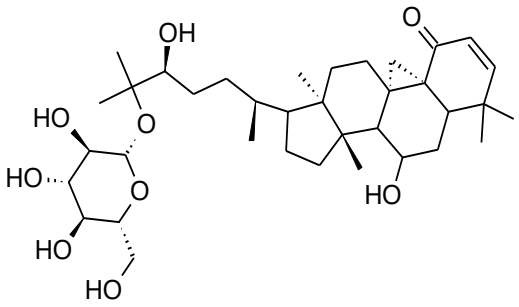
[38]: Sutherlandioside A



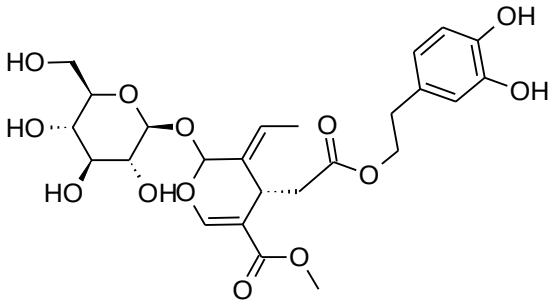
[39]: Sutherlandioside B



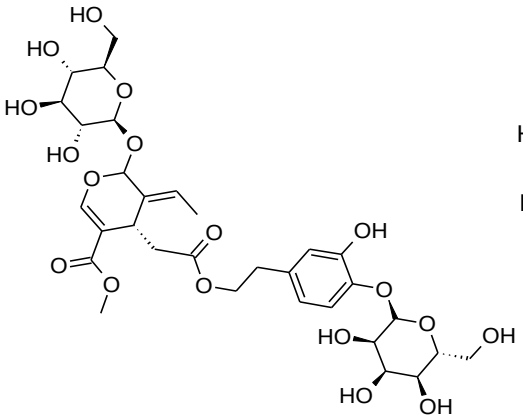
[40]: Sutherlandioside C



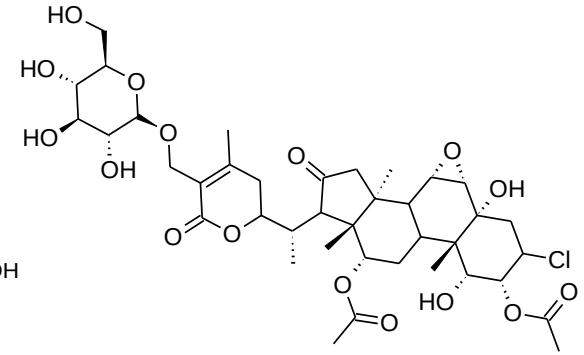
[41]: Sutherlandioside D



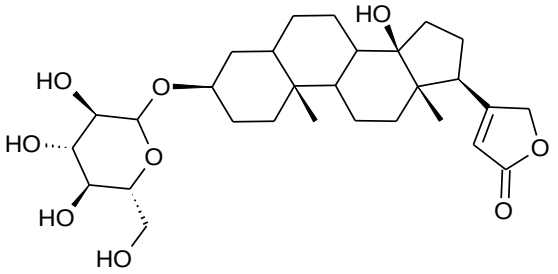
[42]: Oleuropein



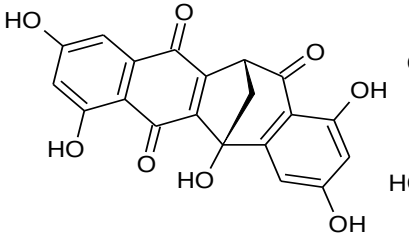
[43]: Oleuropein monoglucoside



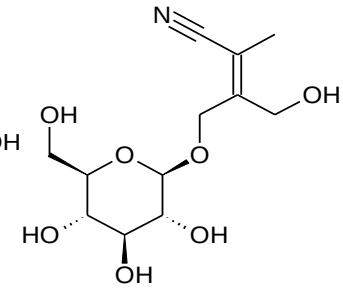
[44]: Plantagiolide



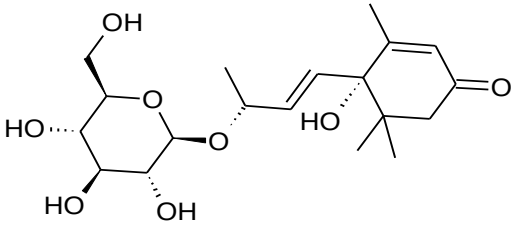
[45]: Digitoxigenin-glucoside



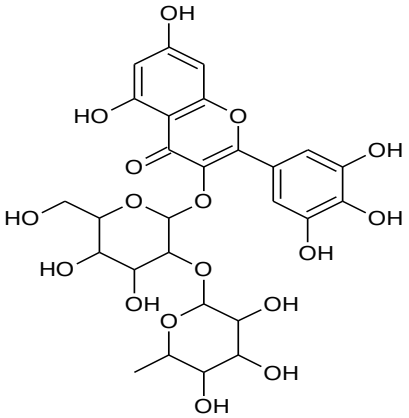
[46]: Natalenone



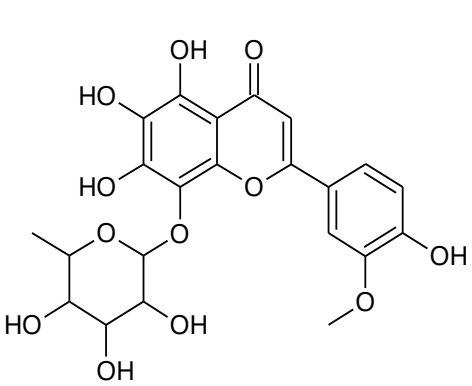
[47]: Sutherlandin (A-E)



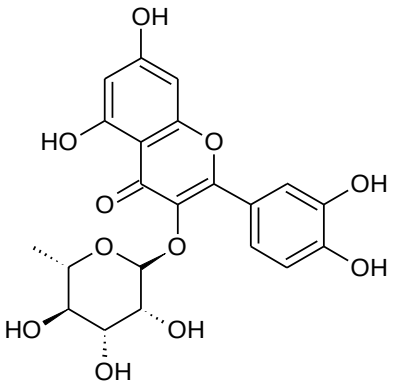
[48]: Roseoside



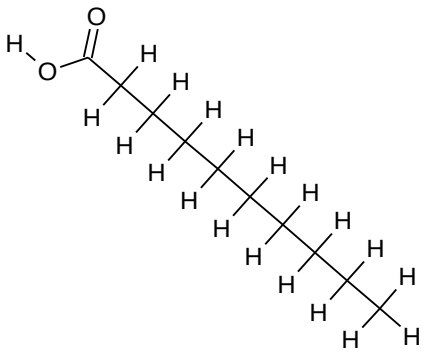
[49]: Myricetin-3-neohesperidoside



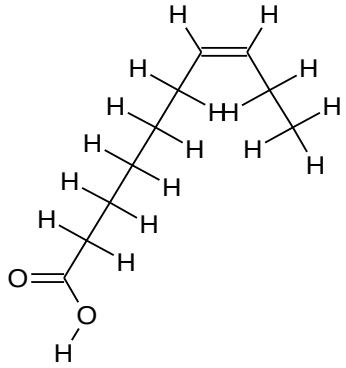
[50]: Estragonoside



[51]: Quercitrin



[52]: Lipid Saturated



[53]: Lipid Unsaturated

Figure 4.7 Chemical structures of compounds tentatively identified in eight selected antiviral extracts by UHPLC-qTOF-MS

Other compounds that were detected in negative ionisation mode include sutherlandioside A-D, and sutherlandin A-D present in *S. frutescens* which are documented for biological activities such as anti-cancer, anti-microbial, antiviral, anti-inflammatory, anti-therosclerotic, anti-oxidant, anti-thrombogenic, and anti-allergic properties (Umesh and Jamsheer, 2018). A quercetin-derived flavonol, quercetin-3-O-robinobioside (Q3R) was tentatively identified at *m/z* 771.1982 present in *A. digitata*. Q3R from the aerial parts of *Houttuynia cordata* was evaluated against influenza virus using a cytopathic effect (CPE) reduction assay. From this study, Q3R reduced the influenza virus at concentrations of 100 and 10 µg/mL by 86 % and 66 %, respectively (Choi et al., 2009). Quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside and kaempferol, potent antiviral flavone glycosides from the leaves of *Ficus benjamina*, were reported to have strong inhibitory activity against HSV-1. These compounds exhibited EC_{50} values of 1.5, 3.0, 0.9 and 25.0 µM, respectively, with the positive control acyclovir having an EC_{50} value of 0.1 µg/mL (Yarmolinsky et al., 2012).

Previous studies on the mechanism of action of metabolites profiled in this study have been reported. The molecular docking of ferulic acid was evaluated against the Tobacco mosaic virus (TMV)-coat protein (TMV-CP). This study found that ferulic acid interacts with tyrosine at position 72, valine, serine, that are involved in the self-assembly of the TMV-CP. Results demonstrated that ferulic acid had binding affinity with a Kd value of 12.6 µM and formed strong hydrogen bonds most amino acids, leading to disease inactivation and antiviral activity (Ren et al., 2020). A study of the antiviral activity of chlorogenic acids against Influenza H7N9 A/Anhui/1/2013 and A/Shanghai/1/2013 outlined on the mechanism of actions involved in inhibiting the virus. This was determined on the neuraminidase's enzyme showed the binding ability of neuraminidase A/Anhui/1/2013 for oseltamivir carboxylate and quercetin. The binding affinity for oseltamivir carboxylate, oleanolic acid, baicalein, quercetin, and chlorogenic acid were greater than −6.91 kcal/mol. It was evident that chlorogenic acid, quercetin, and baicalein binds to neuraminidase by the formation of H-bonds. However, neuraminidase A/Shanghai/1/2013 formed 17 H-bonds with chlorogenic acid which suggest that chlorogenic acid has potent neuraminidase inhibitory effects (Liu et al., 2015). Linoleic acid was shown to form a H-bond with ARM-III of Glu343 and therefore playing an important role as an agonist for PPARγ (Guasch et al., 2011). This literature review on the possible mechanism of polyunsaturated fatty acids highlights that fatty acids act as agonists that influence mostly receptor proteins which leads to the disruption of disease progression.

4.4 Conclusion

In this chapter, ^{1}H-NMR-based metabolomics coupled with multivariate statistical analysis was applied to investigate metabolites in 20 medicinal plant extracts which possess anti-RVFV activity. The results illustrated that ^{1}H-NMR-based metabolomics is capable of discriminating classes of metabolites that attribute to the antiviral properties of selected medicinal plants. According to the contribution plot, the aliphatic and sugar regions were positively associated with activity of the extracts while the aromatic region might have played a role in activity by providing synergistic effects. Furthermore, annotation and putative identification of compounds of the spectral regions that contributes the activity showed the presence of classes of compounds such as terpenoids, flavonoids, alkaloids, fatty acids and amino acid type of compounds. Using UHPLC-qTOF-MS confirmed the annotated compounds by ^{1}H-NMR, and additionally putatively identified a number of compounds in the active samples. These include various chlorogenic acids, amino acids and flavonoids. The focus was however on the compounds present in all the active samples, as these compounds are proposed to significantly contribute to the activity of the samples. Of importance in this study, therefore, is the annotation of two oxygenated fatty acids profiled for the first time in all active samples. These compounds have been reported to exert strong antiviral activity against various viruses with other beneficial activities including anti-inflammatory and anti-oxidant.

In general, metabolomics is a technique that can aid in differentiating biological samples to identify metabolites that play a significant role in the biological activity. This study presents the first report of using ^{1}H-NMR-based metabolomics to identify the anti-RVFV metabolites in plants belonging to different families consisting of complex mixtures of compounds. A combination of different analytical techniques is however important to provide insight into the complexity of the chemical profiles of plants. The results obtained in this study indicates that hydroxy polyunsaturated fatty acids from eight tested plant extracts, may be related to the antiviral potency observed against the RVFV. An extensive literature search was conducted to better understand the mechanism of action of plants and their constituents. With two fatty acids, 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid and 13-hydroxy-9Z,11E-octadecadienoic acid found in all samples that showed potent antiviral activity and having hydroxy-linoleic acid chemical skeleton, it can be suggested that their mechanisms of antiviral activity is endogenous. Literature strongly supports that hydroxy-linoleic acid interferes with the functions of the nuclear receptor proteins such as PPARγ and the agonistic behaviour of

polyunsaturated fatty acids may lead to viral inactivation. A future research objective is to elucidate the precise metabolic pathways of significant anti-RVFV compounds which will enable more insight to guide drug discovery and development processes.

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Chapter 5

General overview and discussion

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5.1 Discussion

Arthropod-borne viruses (arboviruses) are a cause of animal and human diseases worldwide and are on the rise in developing countries. Arboviruses require an arthropod vector in their transmission cycle, in which they must replicate before transmission. Arthropod vectors, including ticks, mosquitoes, midges and flies transmit diseases during blood meals to the hosts (McMillen & Hartman, 2018). *Aedes aegypti* mosquitoes are the most important arboviral vectors transmitting viruses including rift valley fever virus (RVFV), yellow fever virus (YFV), dengue virus (DENV), and chikungunya virus (CHIKV) (Saldaña, Hegde & Hughes, 2017). Arbovirus emergence and re-emergence are influenced by several parameters, including the changing anthropological behaviour, the high mutation rate, temperate tropical climates, high rain precipitation and geographical aspects (Liang, Gao & Gould, 2015). From the family Bunyaviridae comes a genera *Phlebovirus* where RVFV is the type species (Pepin et al., 2010; Spiegelet et al., 2016). RVFV causes severe morbidity, including hepatitis, haemorrhagic fevers in humans and in animals impaired embryonic development is typical. RVFV outbreak has been reported in and outside Africa (Pepin et al., 2010) with a severe impact on livestock losses and human morbidity. Despite significant efforts to combat the (re) emergence of the pathogen, there is little therapeutic development.

The traditional use of medicinal plants is a strong field of indigenous knowledge systems widely practiced by native people of South Africa. Recent pharmacological studies of medicinal plants have proven to provide drug leads that are vital in the development of new therapeutic agents (Süntar, 2019). Medicinal plants typically contain a mixture of secondary compounds that work individually as a single entity or in synergy to combat ailments (Ayseli et al., 2016). Compound classes such as phenolic acids, flavonoids, terpenes, alkaloids, and saponins have shown several biological properties, including anti-bacterial, anti-fungal, antiviral, anti-inflammatory, and antioxidant and are being widely investigated in drug discovery and development (Chandrasekara and Shahidi, 2018). Scientific reports have shown an increasing number of publications investigating the efficacy of medicinal plants which have positive effects on the improvement of health both in humans and animals.

In this study, plants selection was based on published literature on the ethnopharmacological uses and pharmacological properties against various viruses for

bioprospecting of anti-RVFV activity. Twenty selected plants were extensively extracted with 50% aqueous methanol in order to obtain a lucrative number of polar constituents in an extract. The minimum non-toxic concentration (MNTC) was determined by evaluating the cytotoxic effects of the extracts on Vero cells using the MTT assay prior to determining the anti-RVFV activity of the extracts. Extracts were found to be relatively non-toxic with LC_{50} values > 20 µg/mL. The cytotoxicity results were compared to the standard positive control, doxorubicin, which possessed an LC_{50} value of 10 µg/mL which is indicative of toxic effects.

In addition to the conventional *in-vitro* cell monitoring assays such as the MTT colorimetric method, RTCA has proven to be an innovative technology to obtain information on the behaviour, dissemination, and the well-being of cells in the assessment of antiviral activity. Selected plants exhibited good to moderate antiviral activity against RVFV. However, eight out of twenty selected plants showed potency against the virus. Our study has demonstrated that medicinal plants tested have a viral-induced cytopathic cellular protective ability. The best antiviral activity was obtained with the *E. croceum* extract which significantly decreased the number of $TCID_{50}$ up to 10^2 log of the RVFV infectivity followed by *A. afra* and *A. digitata* with a reduction of up to 10^4 log. An average potency compared to *E. croceum* was observed in *E. transvaalesis, E. natalensis, H. aureonitens,* and *S. frutescens* which lowered the RVFV viral load when the virions were subjected to 100 µg/mL of the extracts which had the activity range of 10^4 - 10^5 of $TCID_{50}$.

Oxidative stress is among the main areological agents that are involved in the development of degenerative diseases and viral infections. Oxidative stress has been implicated in triggering an inflammatory immune response which leads to overproduction of ROS. This phenomenon occurs when free radicals such as hydrogen peroxide, superoxide anion, and hydroxyl radicals are not quenched by antioxidative systems (Xu et al., 2017). Oxidative stress plays a role in the pathogenesis of many acute ailments, including viral induced diseases (Yao et al., 2020). Several studies have shown that surplus ROS in viral infected patients disrupted cellular processes and encouraged viral replication. However, the opposite was true with patients on an antioxidant regime (Gonçalves et al., 2017; Bellavite and Donzelli, 2020). Therefore, consumption of an antioxidant-rich diet plays a crucial role in preventing and protecting cellular oxidative stress in humans (Jin et al., 2014; Iranshahi et al., 2015; Xu et al.,

2017). Plants investigated in this study strongly inhibited the DPPH and $ABTS^+$ radicals and reduced the production of LPS-induced reactive oxygen species. Mechanisms of antioxidants and oxidants may modulate excessive ROS/RNS production and oxidative stress in living cells, and this would be a promising strategy for the treatment of viral-induced inflammatory disorders. Eight extracts with the best antiviral activity were screened for their free radicals scavenging potential. These extracts significantly inhibited the DPPH and $ABTS^+$ free radicals. Among eight tested extracts against DPPH radical, five extracts exhibited EC_{50} values < 10 µg/mL with *A. digitata* (EC_{50} = 4.64 µg/mL) and *E. natalensis* (EC_{50} = 5.30 µg/mL) showing the best activity and positive control, ascorbic acid showed results significant to those of the extracts with EC_{50} = 2.50 µg/mL. It was observed that the DPPH scavenging activity of the extracts seemed to correspond directly to the $ABTS^+$ scavenging activity with *E. croceum*, *E. natalensis* and *A. digitata* exhibiting the best activity with an EC_{50} = 4.12, 5.00, 5.04 µg/mL, respectively. It is worth noting that the observed high $ABTS^+$ scavenging activity of the extracts has comparable significant EC_{50} values to the positive control, ascorbic acid which exhibited good $ABTS^+$ reducing power with an EC_{50} value of 2.30 µg/mL. Additionally, extracts also showed the reduction of LPS-induced ROS and RNS with high activity > 60 % by *E. croceum*, *E. natalensis* and *A. digitata* in both the ROS and RNS assays. There is evidence from the literature that demonstrates an increase of ROS/RNS in living cells as the main conditions of severe disease, persuades the investigation for plant compounds that can reduce ROS/RNS to required levels during viral infection. Plant extracts might have different mechanisms when inhibiting viral infections, however, such properties could act by reducing the ROS production and further assist in other modes of therapeutic development.

Metabolomics provides crucial information on the phytochemical processes in a biological sample. This technique comprises a multi-platform system that employs analytical instruments such as NMR, GC-MS and UPLC-TOF-MS, and multivariate statistical tools to enhance predictability and discrimination of samples. Moreover, annotation methods are employed to amplify the elucidation of metabolites of significance. This study used ^{1}H-NMR-based metabolomics coupled with multivariate statistical analysis to demonstrate the ability of the model to differentiate diverse classes of metabolites in plant extracts which are responsible for the antiviral activity.

The unsupervised pattern recognition analysis (PCA) was applied to give an overview of the samples. The PCA did not reveal clear discriminative patterns of the samples in relation to the activity. However, the hierarchical cluster analysis (HCA) dendrogram was developed to evaluate whether some groupings from the data can be generated. The HCA dendrogram grouped subjects with similar features into three clusters, similarly to the PCA analysis. Subsequently, an OPLS-DA score plot was created, and it showed significant discrimination between the active and non-active samples with an R^2X value of 0.830 and a Q^2 value of 0.706. The HCA analysis confirmed the separation of the samples into two groups.

Validation was performed to evaluate the classification performance of the model. The receiver operated characteristic (ROC), which calculates the area under the curve (AUC) was plotted and the cross-validated predictive residual (CV-ANOVA, *p*-value < 0.05) was performed. To further validate the model the 100 permutation tests with an $R^2X = 0.851$ and $Q^2 = 0.561$ was observed. The ROC (AUC) = 0.9980 with a *p-value* < 0.05 was obtained. The validation methods confirmed that the model showed good reliability and predictability accuracy.

Analysis of the loading S-plot and VIP score plot, identified the ^{1}H-NMR regions that are positively associated with antiviral activity. The chemical shifts of the VIP scores > 1 were considered significant. A further selection of variables was done using the loading S-plots which helped to identify discriminating variables between the groups. The loading S-plots demonstrated that variables on the two extreme ends of the S-plot are discriminative. The contribution plot which uses chemical shifts of each sample within different clusters revealed various ^{1}H-NMR bins in the aliphatic and sugar regions as important, with the aromatic region with fewer ^{1}H-NMR bins. These ^{1}H-NMR regions were used to annotate metabolites by matching the ^{1}H-NMR peaks to the Chenomx software and use existing published literature for confirmation of metabolites. The challenge of this work was to be able to elucidate and annotate overlapping peak signals in a spectrum. To overcome this challenge, the UHPLC-MS was used to confirm the presence of compounds.

The UHPLC-qTOF-MS was used as a second analytical platform to tentatively identify and validate acquired annotated data from the same samples. It is hypothesised that the combination of analytical instruments like ^{1}H-NMR and UHPLC-MS enhance the quality of a study and improves the coverage of the metabolome (Marshall and Powers, 2018). Characterization of metabolites using UHPLC-qTOF-MS was applied to analyse and annotate metabolites of active antiviral samples. A targeted approach was followed, and the method yielded 61 metabolites that are prominent in active samples. Classes of compounds characterised include fatty acids, terpenoids, flavonoids, alkaloids, phenolics, and amino acids. These metabolites have been previously reported to exert antiviral potency and other biological activities such anti-inflammatory, anti-diabetes and hepato-protective abilities. Annotated metabolites using Chenomx software and UHPLC-qTOF-MS analysis validate the accuracy of computational software in predicting metabolites of significance. Furthermore, the UHPLC-qTOF-MS analysis led to the identification of two compounds which were present in all active samples. This study reports for the first time the presence of 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid and 13-hydroxy-9Z,11E-octadecadienoic acid in eight tested antiviral plants investigated in this study. Two fatty acid type of compounds 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid and 13-hydroxy-9Z,11E-octadecadienoic acid which are linoleic acid derivatives were shown by the presence of peaks with exact masses of *m/z* 294.22 and *m/z* 296.24 during UHPLC-qTOF-MS analysis. The tentative identification of 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid and 13-hydroxy-9Z,11E-octadecadienoic acid correlates well with ^{1}H-NMR buckets found in the aliphatic and sugar region which are 2.04, 2.12, 2.20, 3.36, 3.68, 3.72, 3.76, 3.92, 3.96, 4.04, 4.24, 4.32, 4.40, 5.88, 5.92, 5.96, 6.0 and 6.40 ppm. Hydroxy fatty acids derived from linoleic acid exhibit a significant biological role in animals and plants. They are renowned for their role in cell signalling and defence in response against pathogens (Li et al., 2016) and *in vitro* they have shown antibacterial, antiviral, anti-inflammatory properties. They have been proven to stimulate several biological pathways that contribute to the activity.

Other interesting metabolites that were annotated include ferulic acid which was present in *A. digitata, A. afra,* and *H. aureonitens.* Chlorogenic acid was present in *A. digitata*, *A. afra, E. transvaalense* and *H. aureonitens*. An alkaloid trigonelline was detected in *A. digitata, E. elephantina* and *S. frutescens*. Caffeoylquinic acids and dicaffeoylquinic acids were abundant in the *A. afra* and *H. aureonitens* extracts. According to our observation, the presence of the latter compounds in plant extracts did not contribute to the clustering of these active samples

on the OPLS-DA but might have played a significant synergistic role in enhancing the activity of samples. However, it can be deduced that the activity of the samples can be attributed to the newly annotated 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid and 13-hydroxy-9Z,11E-octadecadienoic acid compounds. It would however be important to test the compounds for their anti-RVFV activity, cytotoxicity and to elucidate their mechanism of antiviral action.

The study hypothesised that pharmacologically-based selection of plant species with antiviral activity will exhibit significant *in-vitro* anti-RVFV activity and ^{1}H-NMR-based metabolomics coupled with multivariate statistical analyses together with UHPLC-MS can annotate and identify anti-RVFV metabolites from selected medicinal plants. However, using the cytopathic neutralising assay and modern technology such as real time cell analyser (RTCA) method allowed the determination of the antiviral activity of selected plants. Moreover, evaluation of antioxidant scavenging activity, ROS and RNS reduction activity assays proved that plant extracts and their constituents may have different mechanism of viral inhibition. Thus, the overall outcomes of the aims and objectives of this study has scientifically validated the traditional use of medicinal plants and positively proved our hypothesis that selected medicinal plants that exhibit a range of antiviral activities can inhibit the RVFV infection *in vitro* and metabolomics is capable of discriminating and putatively identifying metabolites of significance. Faced with the challenge of resistant strains, especially viral infection and limited therapeutic drugs to fight viral infections, there is a need to investigate the efficacy of medicinal plants *in vitro* and *in vivo* in order to discover new chemical entities to curb viral infections especially emerging/re-emerging viral infection that leads to epidemic outbreaks. Moreover, our study recommends the use of the newly developed technologies such as metabolomic platforms, to accelerate the development and discovery of new drugs. This study provided a glimpse into the antiviral potential of plants-based medicine worth of thorough biological investigation that may lead to drug development and discovery.

The findings in this study validates the ethno-pharmacological and pharmacological uses of medicinal plants, especially the use of *E. croceum, A. digitata, A. afra, H. aureonitens* and *S. frutescens* as they sequentially showed significant anti-RVFV activity. It was surprising that known antiviral compounds such as chlorogenic acids and ferulic acid are not present in the most active extract of *E. croceum*, however, the presence of the two hydroxylated fatty

acids may again suggest the contribution of the activity of these compounds. Synergistic activity might still be possible as quinic acid, with proven antiviral activity is present in *E. croceum*. Moreover, previous studies have shown that glycosylation of secondary compounds influences the biological activities by enhancing their solubility and bioavailability (Xiao et al., 2014). To support this, Rathore et al., (1985) evaluated the effects of digitoxigenin for cardiotonic and Na+/K+-ATPase activity. This study revealed that the glycosylated compounds improves the attachment of compounds therefore elevating the bioactivity of this digitoxigenin compound. Digitoxigen-glucoside isolated from *E. croceum* showed antiviral activity against human immunodeficiency virus (HIV) and Vesicular Stomatitis Indiana virus (VSV) -pseudo-type at low concentrations (Prinsloo, et al., 2010). Therefore, in this study the presence of the glycosylated conjugate type of compounds may have played a role in increasing the anti-RVFV activity of *E. croceum.* However, the stereochemistry of glycosylation is of importance as it determines the activity and toxicity of the compound of which digoxin and digitoxin consisting of tri-glycosylation are examples. Thus, the presence of glucoside moieties in some compounds found in *E. croceum* and other potent anti-RVFV extracts may have contributed to the observed activity. Plants such as *E. croceum*, as guided by the results of this study, can therefore be developed as an anti-RVFV treatment and should also be tested with current treatments to determine its synergistic or additive potential.

Prospectively, we aim at understanding the precise mechanisms of action and metabolic pathways involved in the viral inhibition of newly annotated compounds and comparing their activity to the other identified compounds in this study. Additionally, we envisage investigating the effects of plant extracts and their constituents against viral DNA replication, viral mediators' attachment to cells and immunological activation or suppression mediators of inflammatory response including tumour necrotic factor-α (TNF-α), nuclear factor kappa-B (NF-kB) and cytokines such as prostaglandin E2 (PGE2) in cells. Further, preclinical and epidemiological studies are warranted to authenticate the effectiveness, mechanisms of action of the two novel metabolites involved in the activity, then the possible development of the extracts or pure compounds into a commercial product can be explored. In addition, bioprospecting of combination therapy with the extracts will be developed with RVFV antiviral candidates in the future as well.

5.2 References

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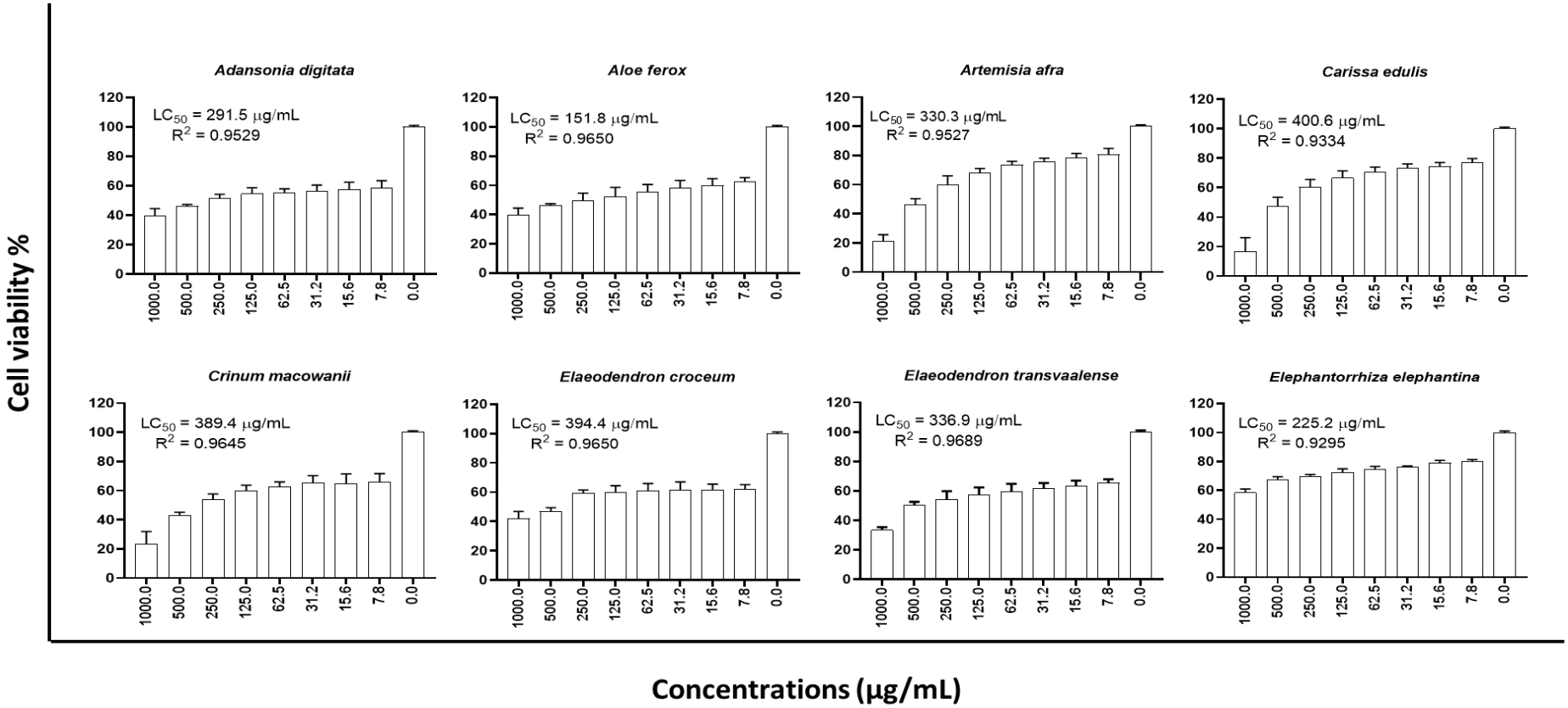
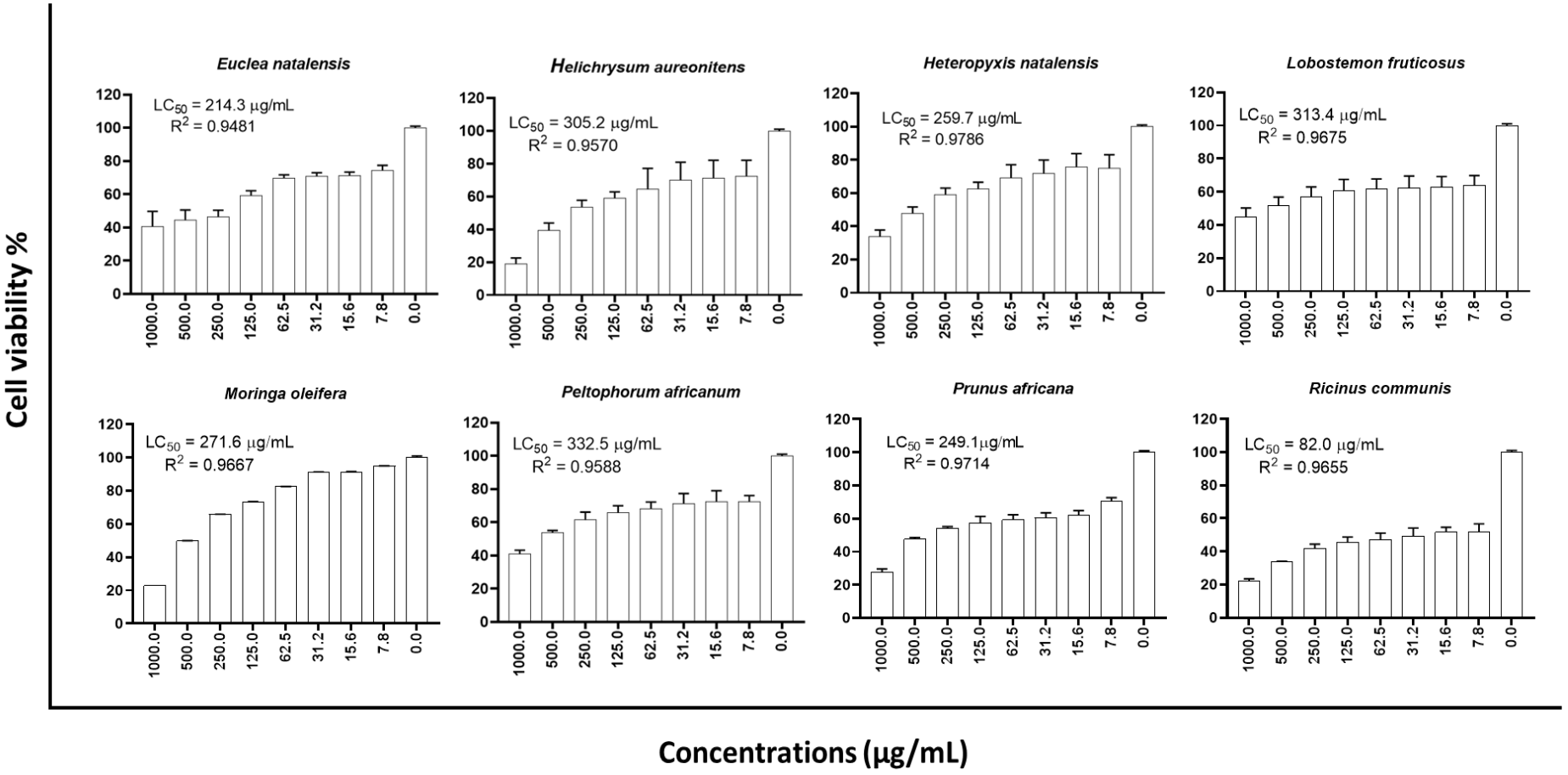
Chapter 6

Appendix

Chapter 6


Cell viability %
Adansonia digitata
LC50 = 291.5 µg/mL
R2 = 0.9529
Aloe ferox
LC50 = 151.8 µg/mL
R2 = 0.9650
Artemisia afra
LC50 = 330.3 µg/mL
R2 = 0.9527
Carissa edulis
LC50 = 400.6 µg/mL
R2 = 0.9334
Crinum macowanii
LC50 = 389.4 µg/mL
R2 = 0.9645
Elaeodendron croceum
LC50 = 394.4 µg/mL
R2 = 0.9650
Elaeodendron transvaalense
LC50 = 336.9 µg/mL
R2 = 0.9689
Elephantorrhiza elephantina
LC50 = 225.2 µg/mL
R2 = 0.9295
0 20 40 60 80 100 120
1000.0 500.0 250.0 125.0 62.5 31.2 15.6 7.8 0.0
Concentrations (µg/mL)


Euclea natalensis
LC50 = 214.3 µg/mL
R2 = 0.9481
Helichrysum aureonitens
LC50 = 305.2 µg/mL
R2 = 0.9570
Heteropyxis natalensis
LC50 = 259.7 µg/mL
R2 = 0.9786
Lobostemon fruticosus
LC50 = 313.4 µg/mL
R2 = 0.9675
Moringa oleifera
LC50 = 271.6 µg/mL
R2 = 0.9667
Peltophorum africanum
LC50 = 332.5 µg/mL
R2 = 0.9588
Prunus africana
LC50 = 249.1µg/mL
R2 = 0.9714
Ricinus communis
LC50 = 82.0 µg/mL
R2 = 0.9655
0 20 40 60 80 100 120
1000.0 500.0 250.0 125.0 62.5 31.2 15.6 7.8 0.0
Cell viability %
Concentrations (µg/mL)

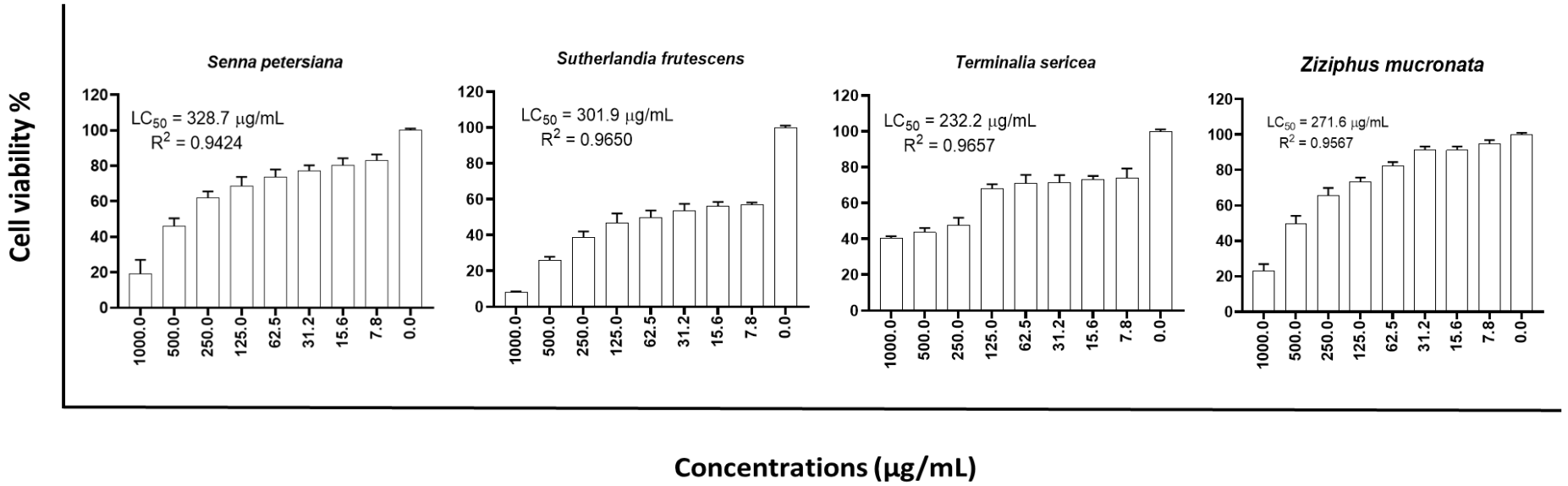
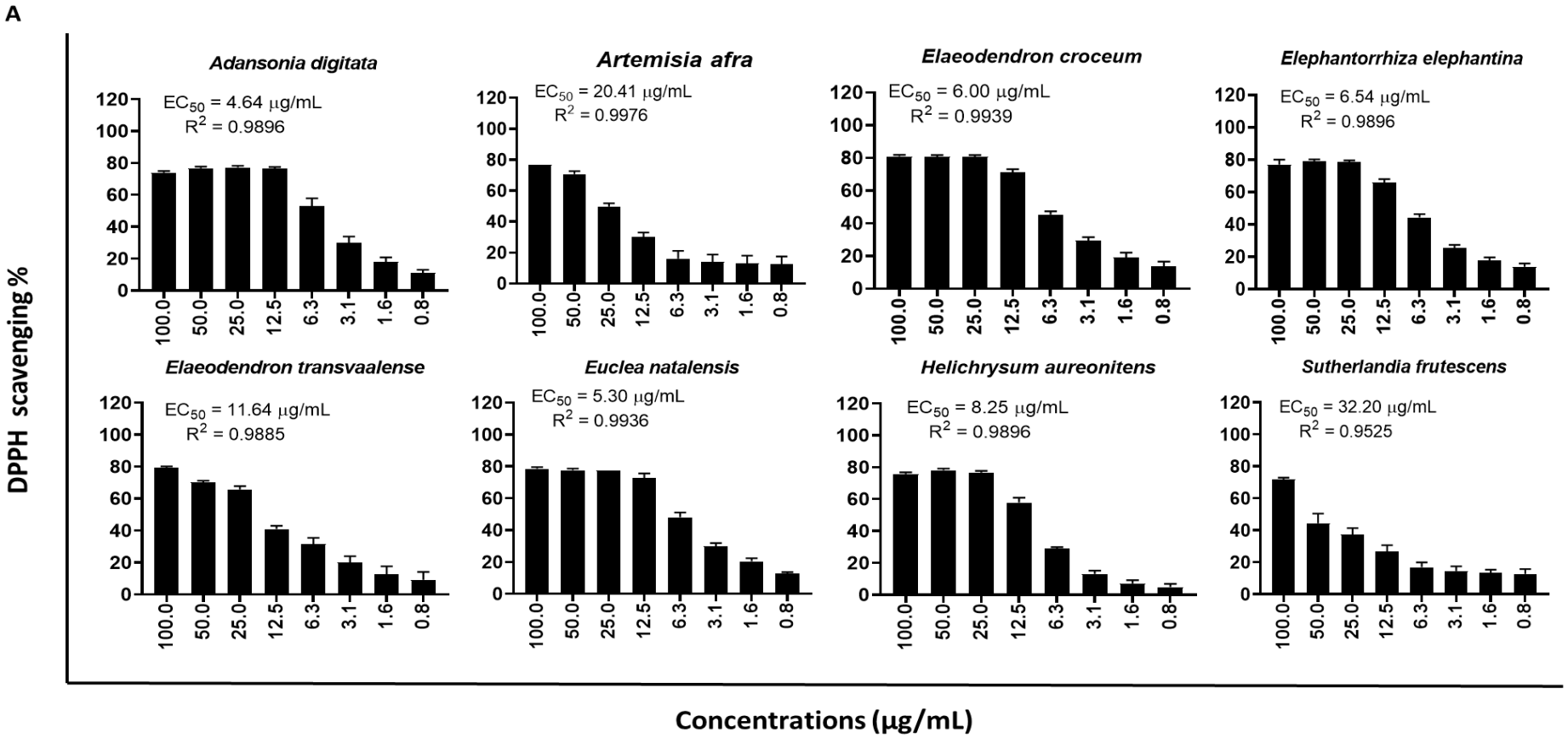


Figure 6.1 Lethal concentration (LC_{50} = µg/mL) of the MTT assays following 48 hours treatment with twenty medicinal plant extracts well known for their antiviral activity.

A


Adansonia digitata
EC50 = 4.64 µg/mL
R2 = 0.9896
Artemisia afra
EC50 = 20.41 µg/mL
R2 = 0.9976
Elaeodendron croceum
EC50 = 6.00 µg/mL
R2 = 0.9939
Elephantorrhiza elephantina
EC50 = 6.54 µg/mL
R2 = 0.9896
Elaeodendron transvaalense
EC50 = 11.64 µg/mL
R2 = 0.9885
Euclea natalensis
EC50 = 5.30 µg/mL
R2 = 0.9936
Helichrysum aureonitens
EC50 = 8.25 µg/mL
R2 = 0.9896
Sutherlandia frutescens
EC50 = 32.20 µg/mL
R2 = 0.9525
DPPH scavenging %
0 20 40 60 80 100 120
100.0 50.0 25.0 12.5 6.3 3.1 1.6 0.8
Concentrations (µg/mL)

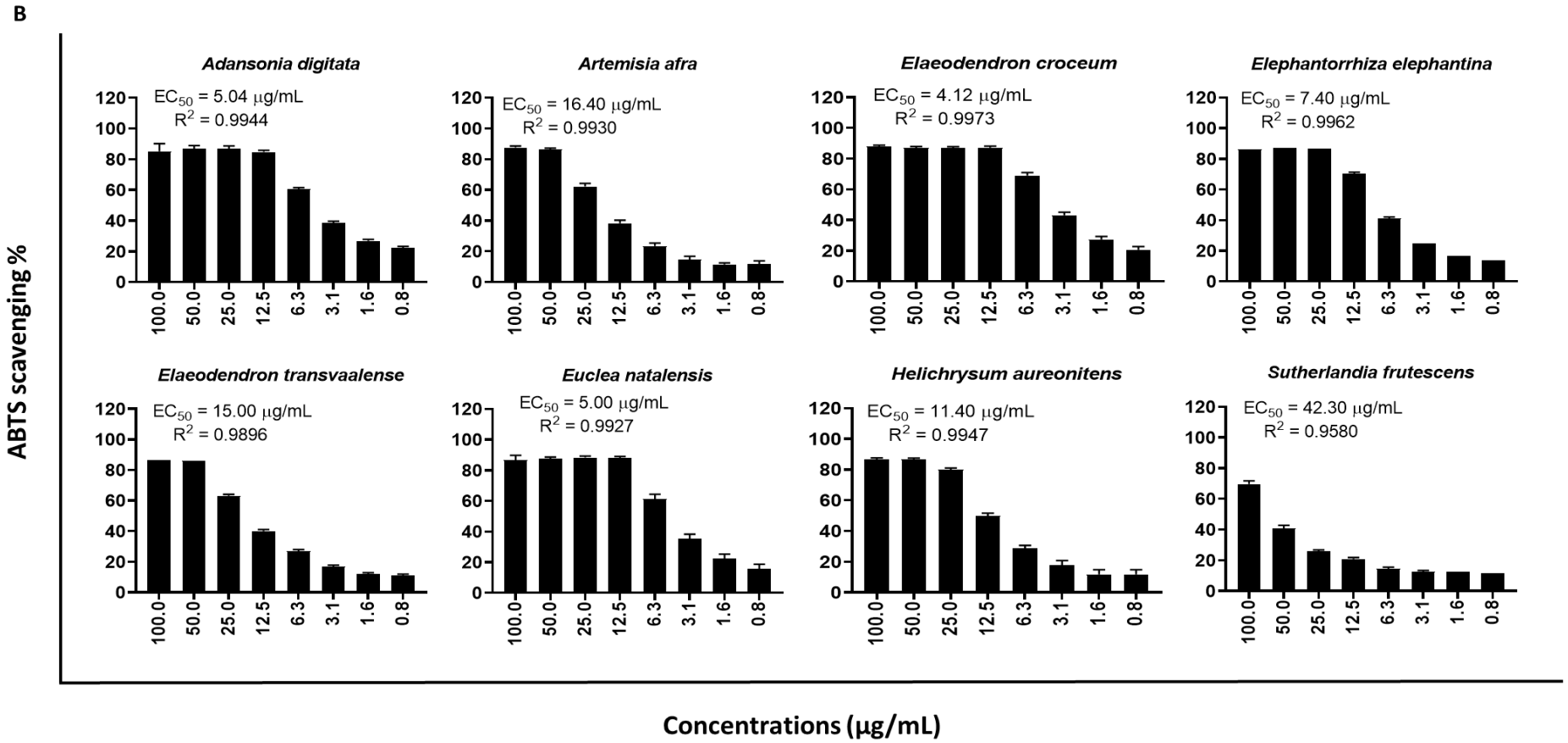


Figure 6.2 Effective concentration (EC_{50} = µg/mL) of the DPPH **(A)** and ABTS **(B)** assays following 48 hours treatment with eight plant extracts well known for their antiviral activity.

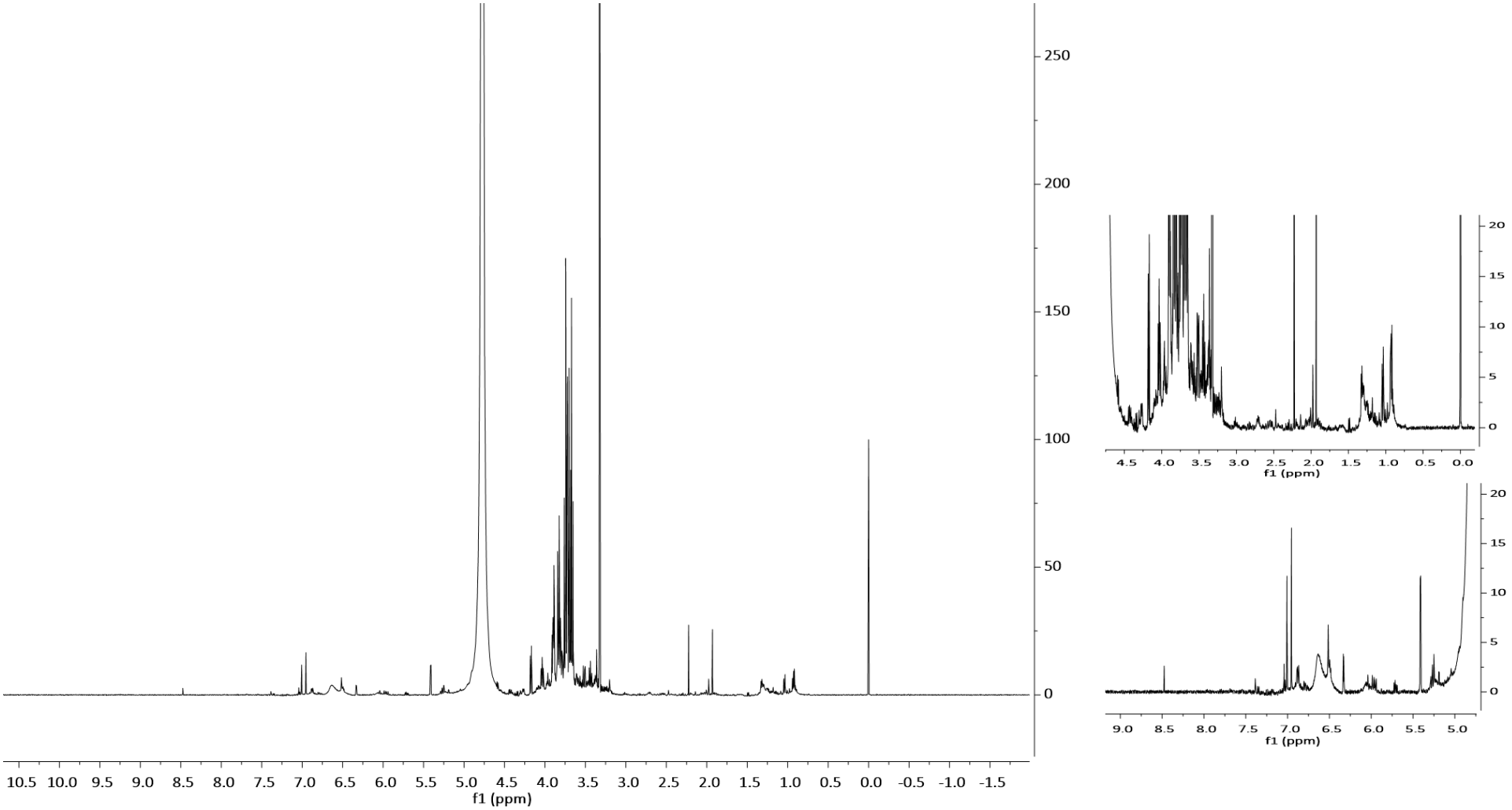


Figure 6.3 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Euclea natalensis* acquired on a 600 MHz NMR.

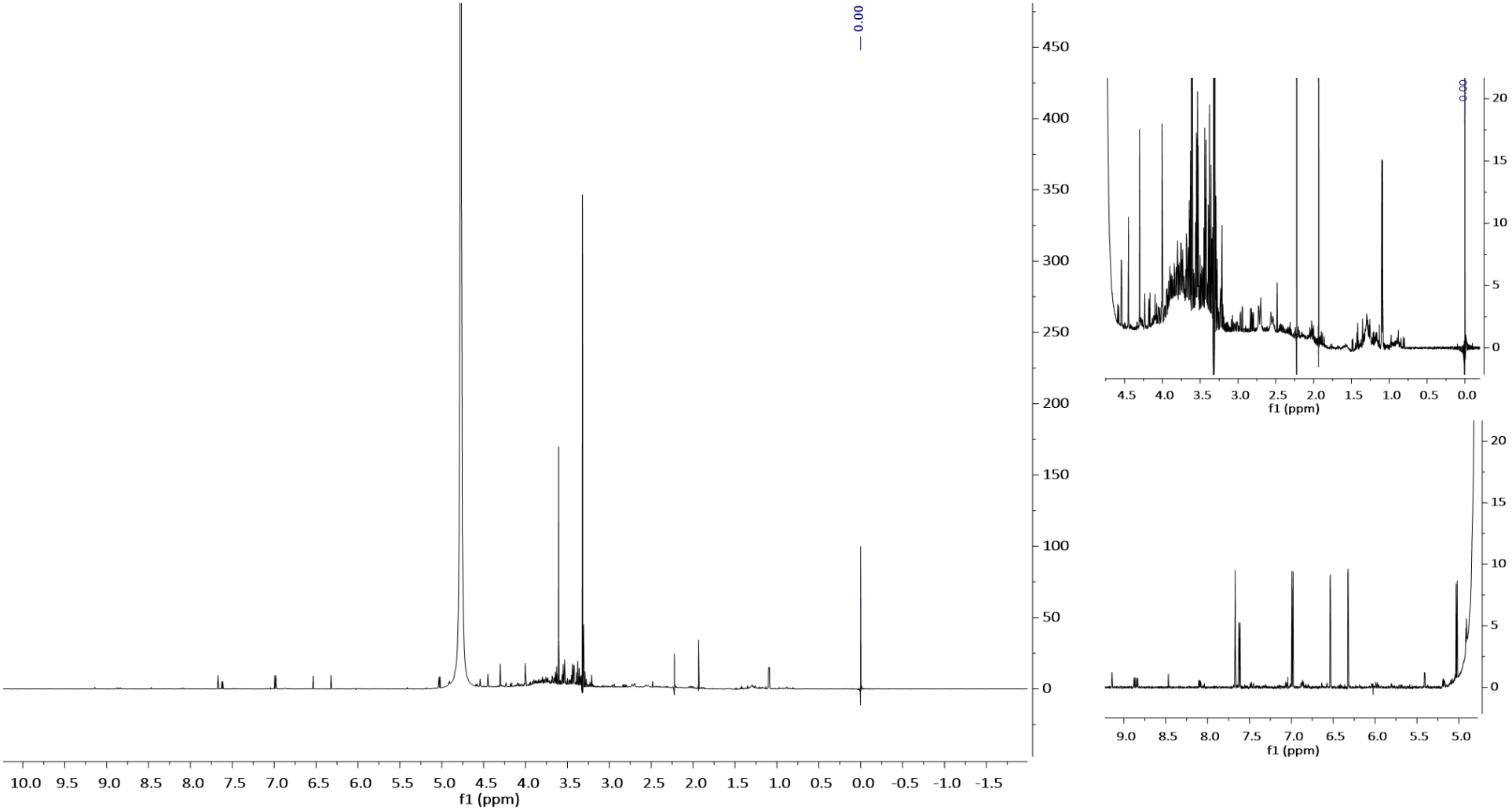


Figure 6.4 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Elephantorrhiza elephantina* acquired on a 600 MHz NMR.

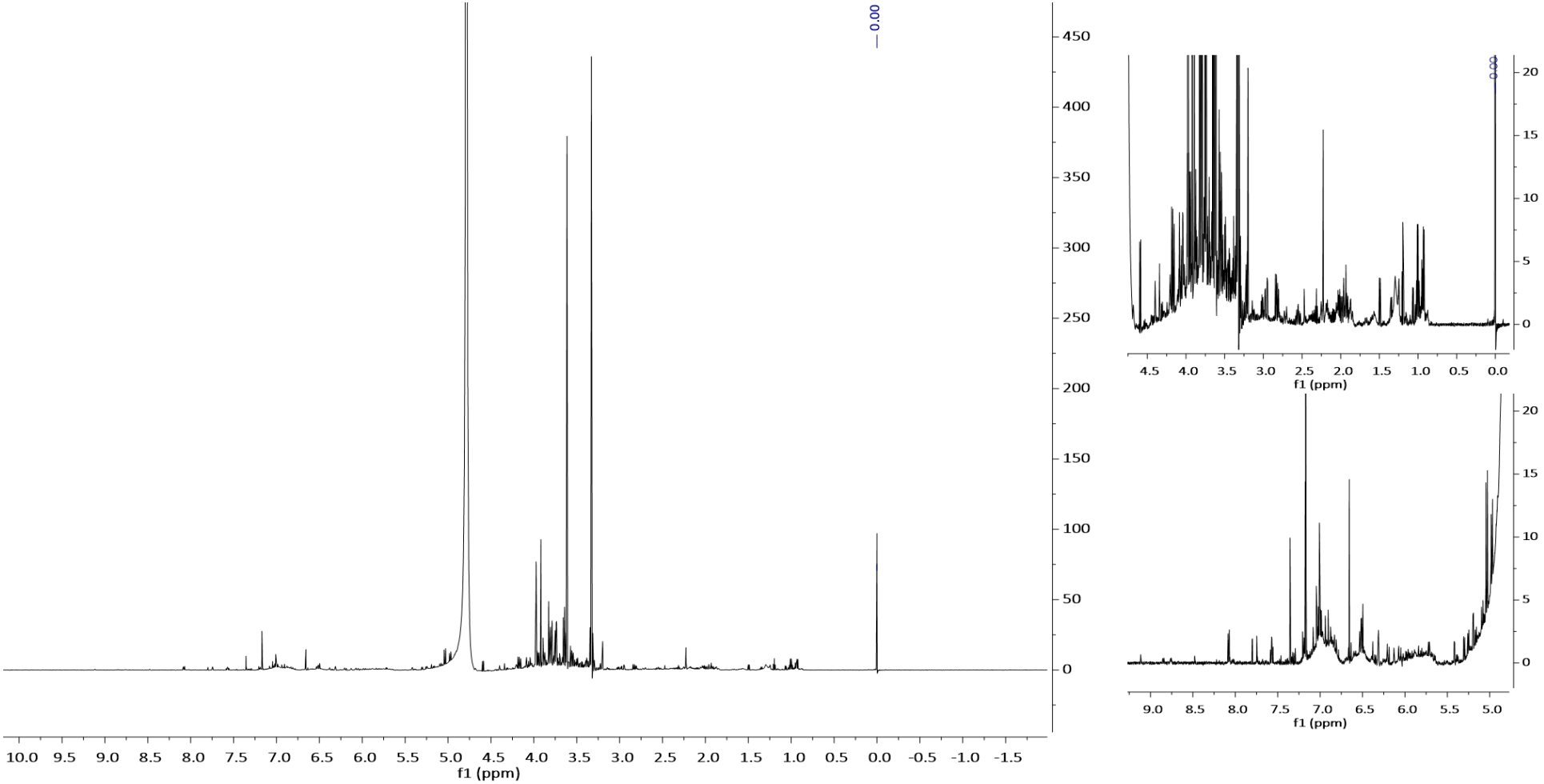


Figure 6.5 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Peltophorum africanum* acquired on a 600 MHz NMR.

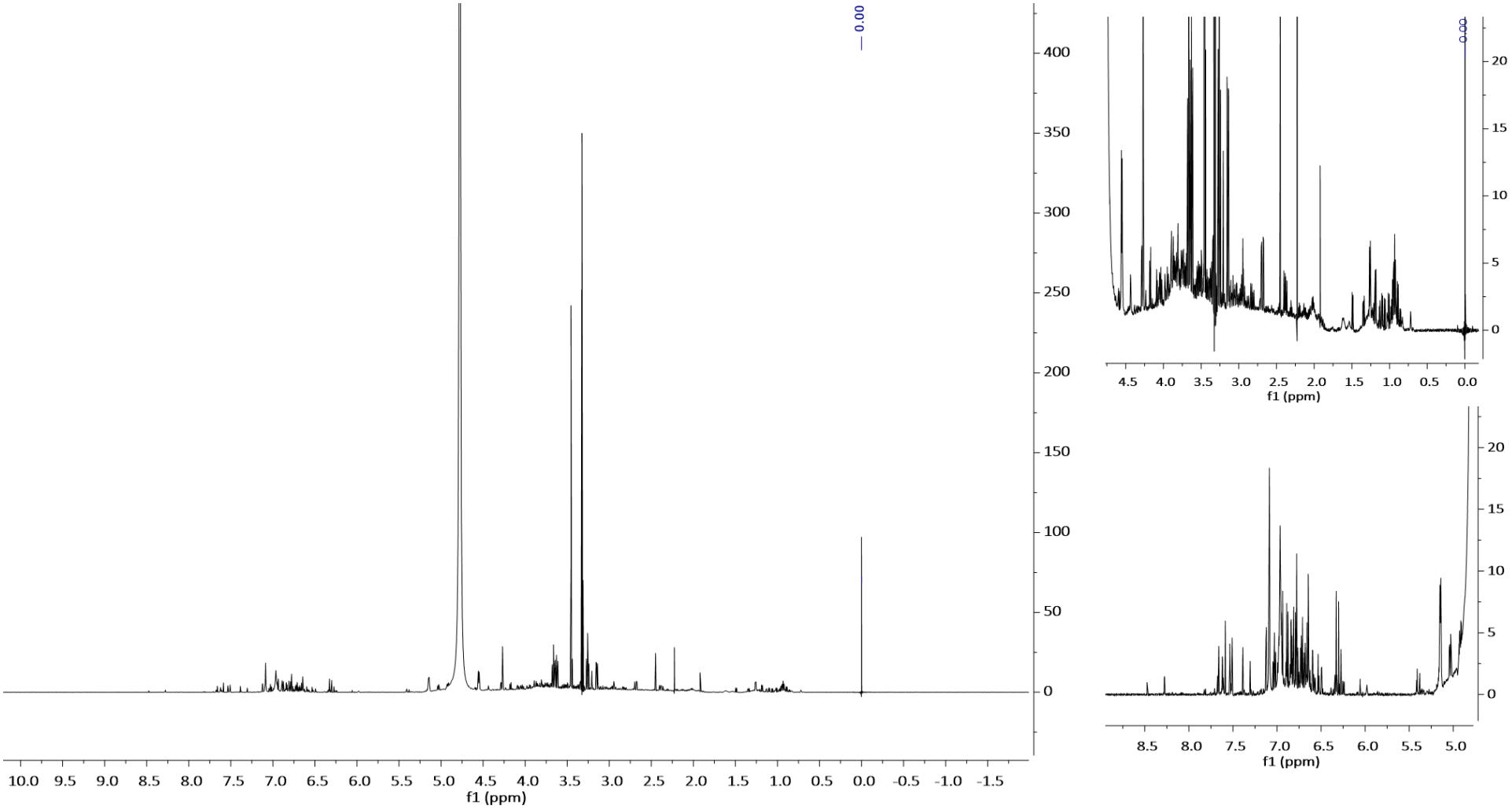


Figure 6.6 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Lobostemon fruticosus* acquired on a 600 MHz NMR.

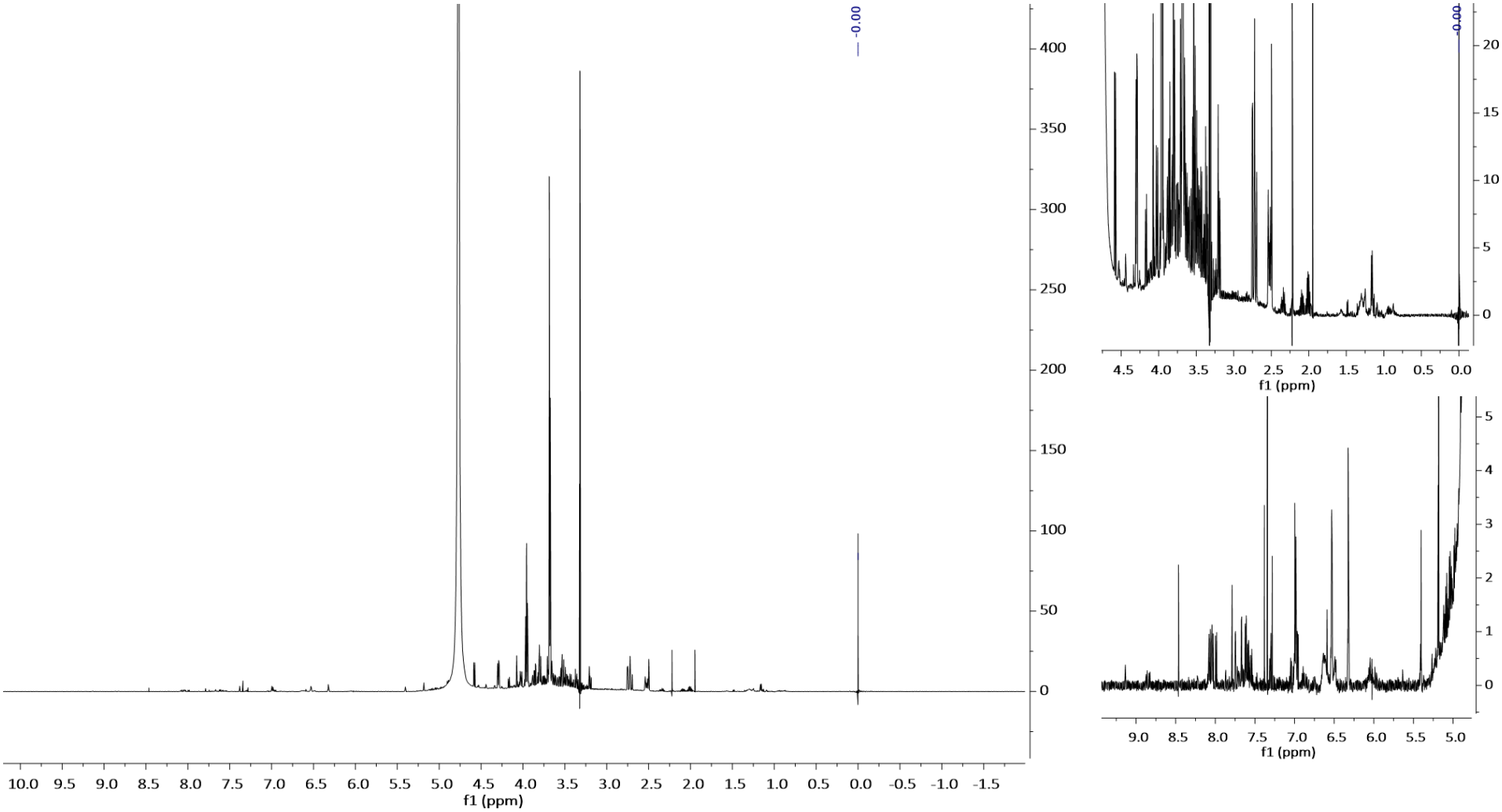


Figure 6.7 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Elaeodendron transvaalensis* acquired on a 600 MHz NMR.

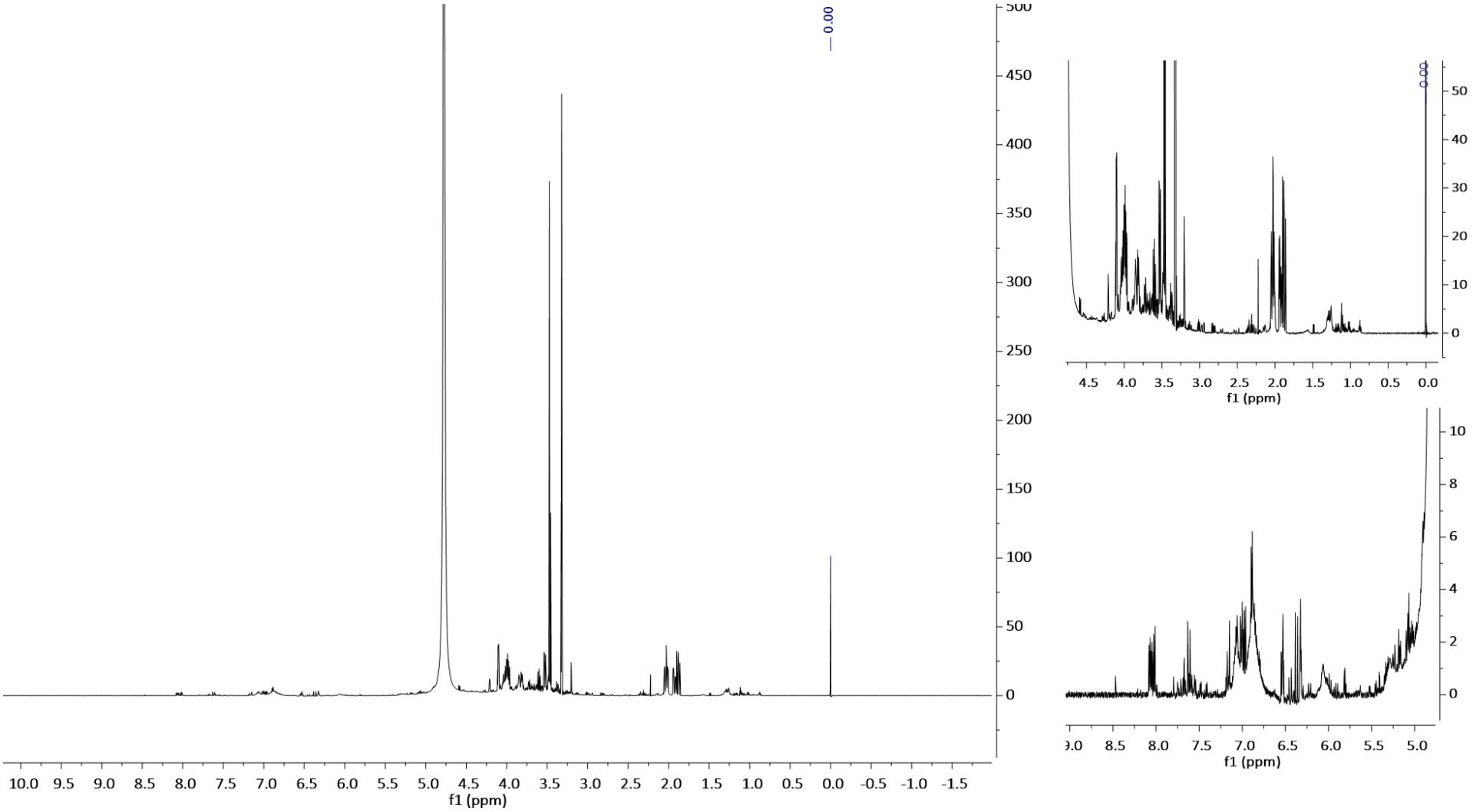


Figure 6.8 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Carissa edulis* acquired on a 600 MHz NMR.

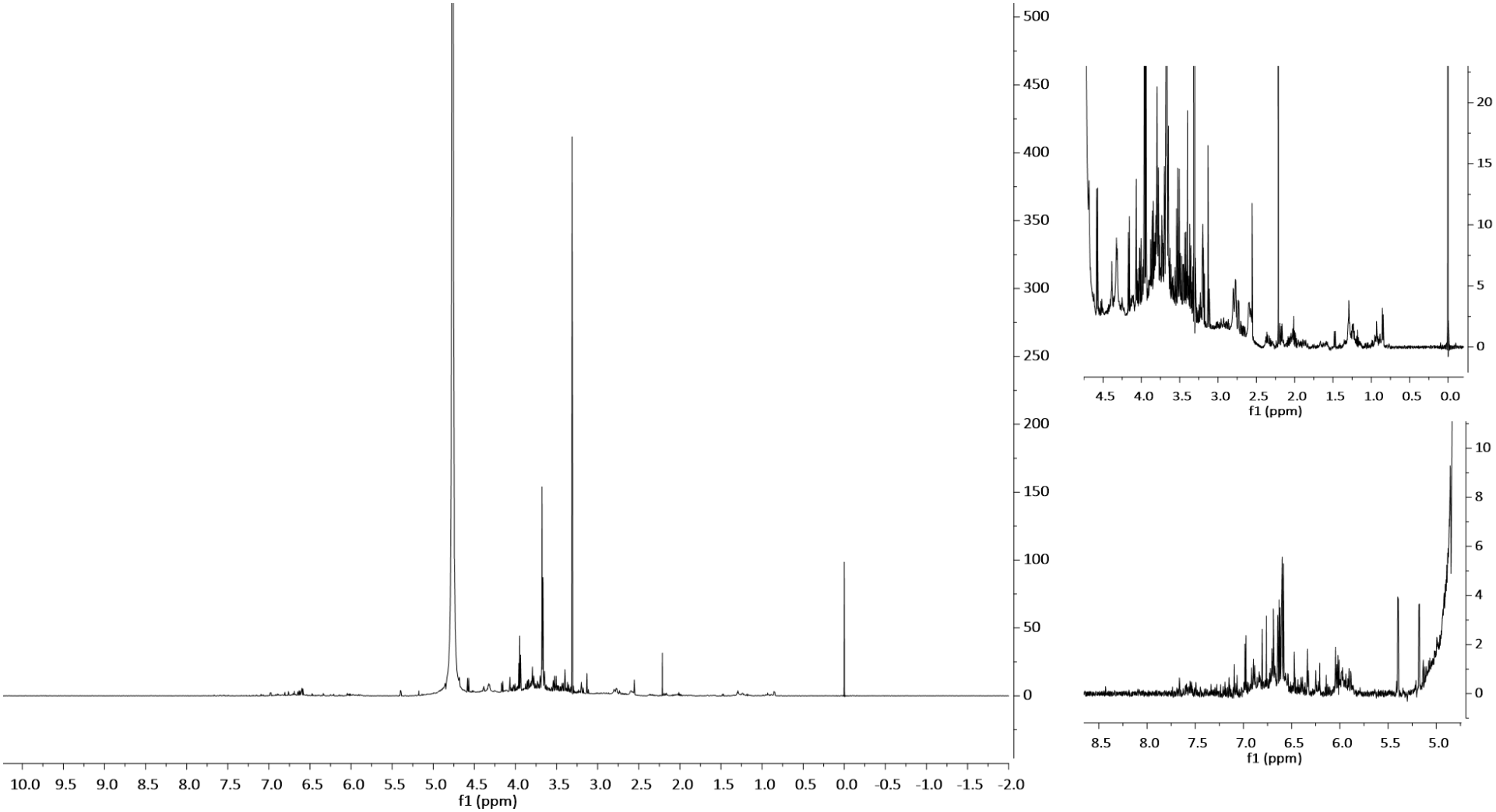


Figure 6.9 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Elaeodendron croceum* acquired on a 600 MHz NMR.

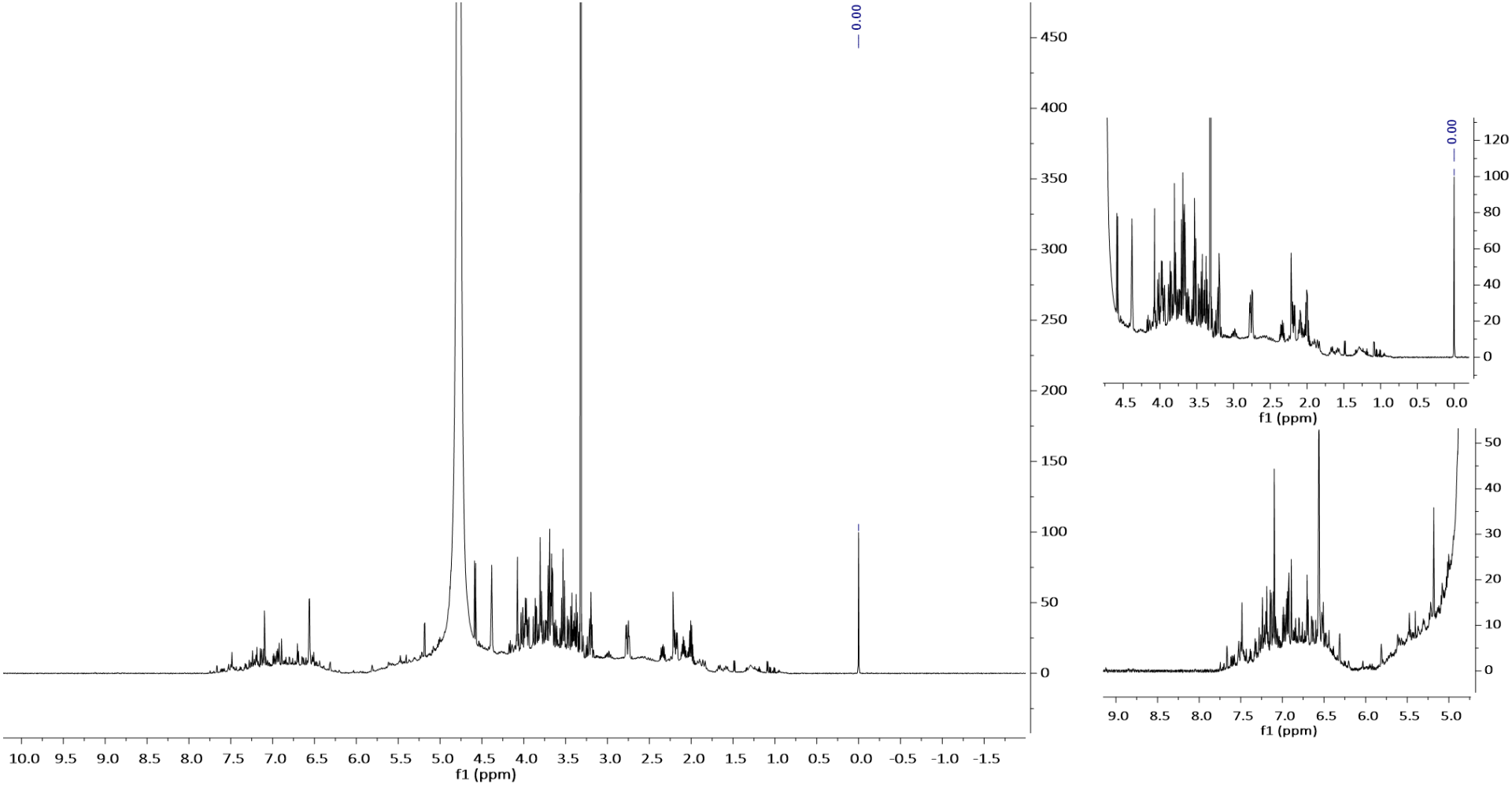


Figure 6.10 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Helichrysum aureonitens* acquired on a 600 MHz NMR.

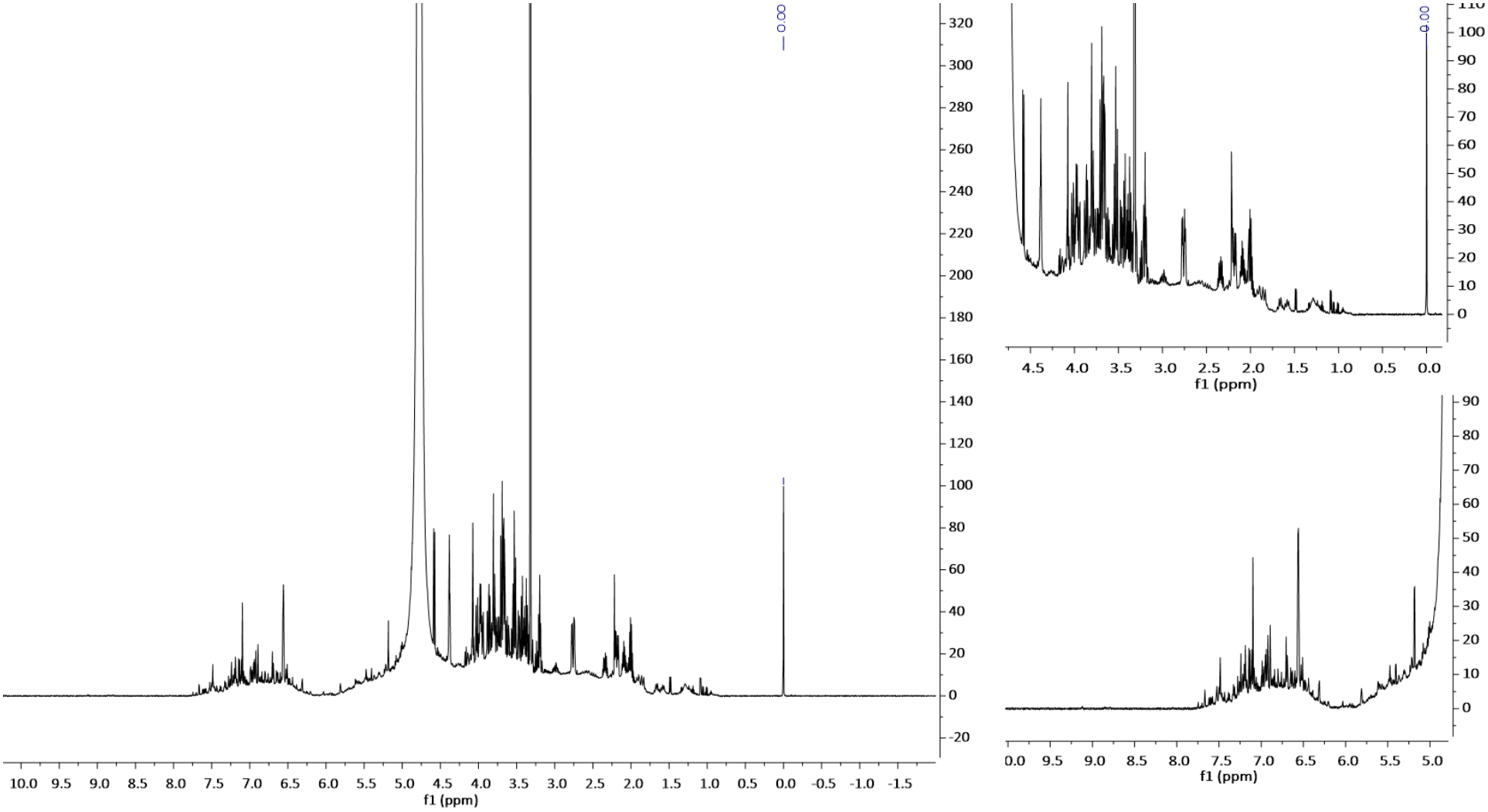


Figure 6.11 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Terminalia sericea* acquired on a 600 MHz NMR.

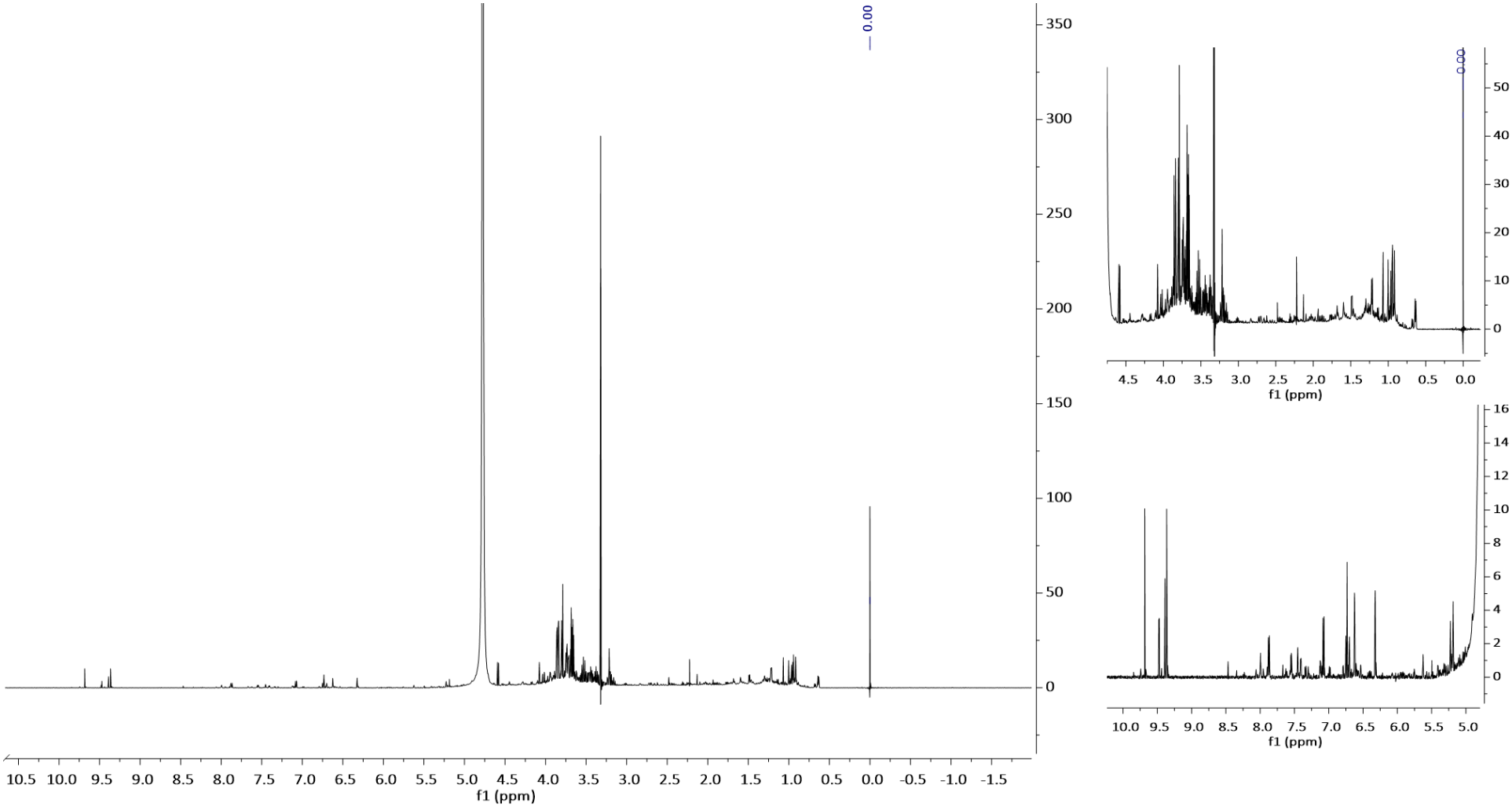


Figure 6.12 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Heteropyxis natalensis* acquired on a 600 MHz NMR.

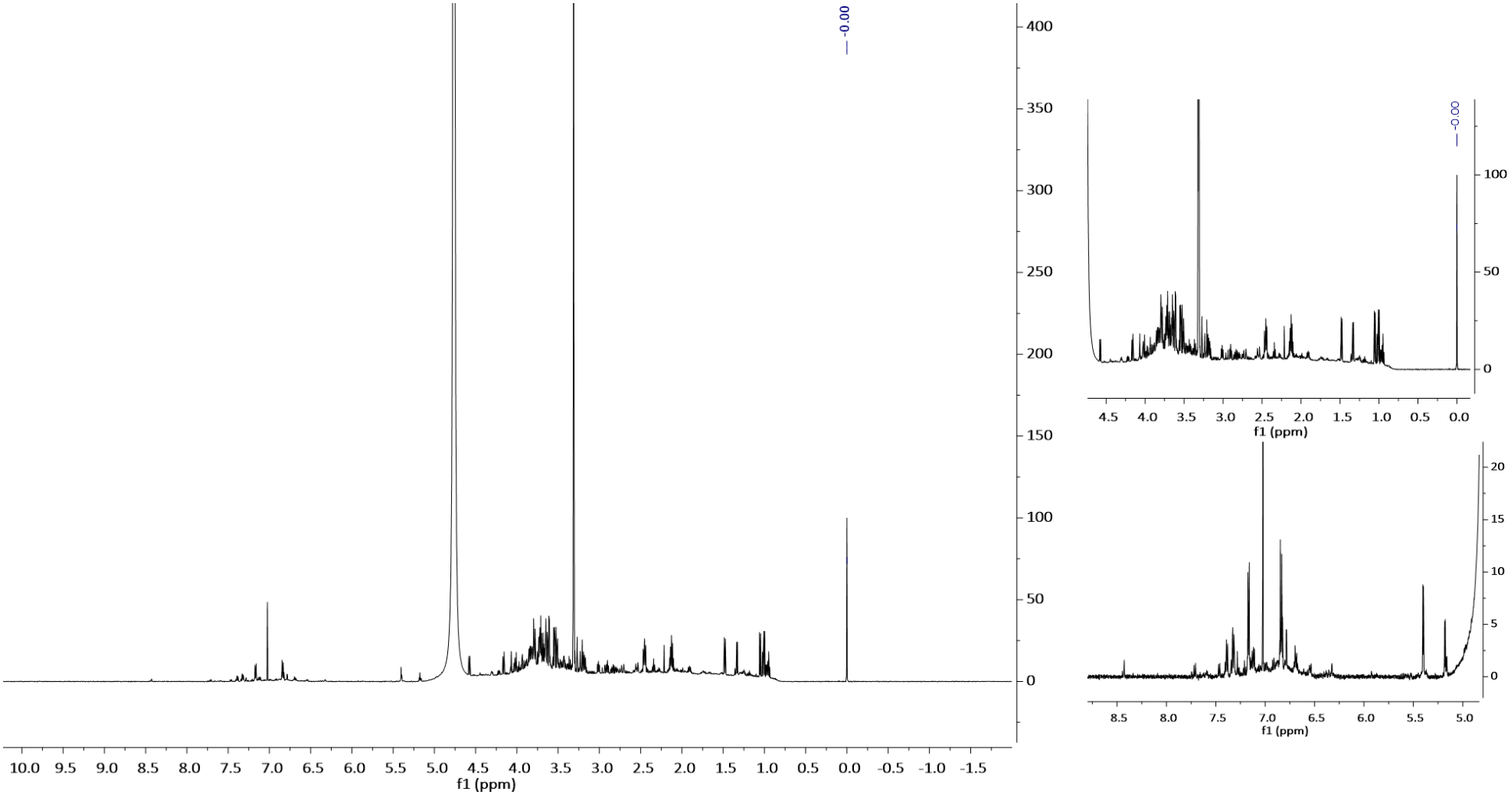


Figure 6.13 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Crinum macowanii* acquired on a 600 MHz NMR.

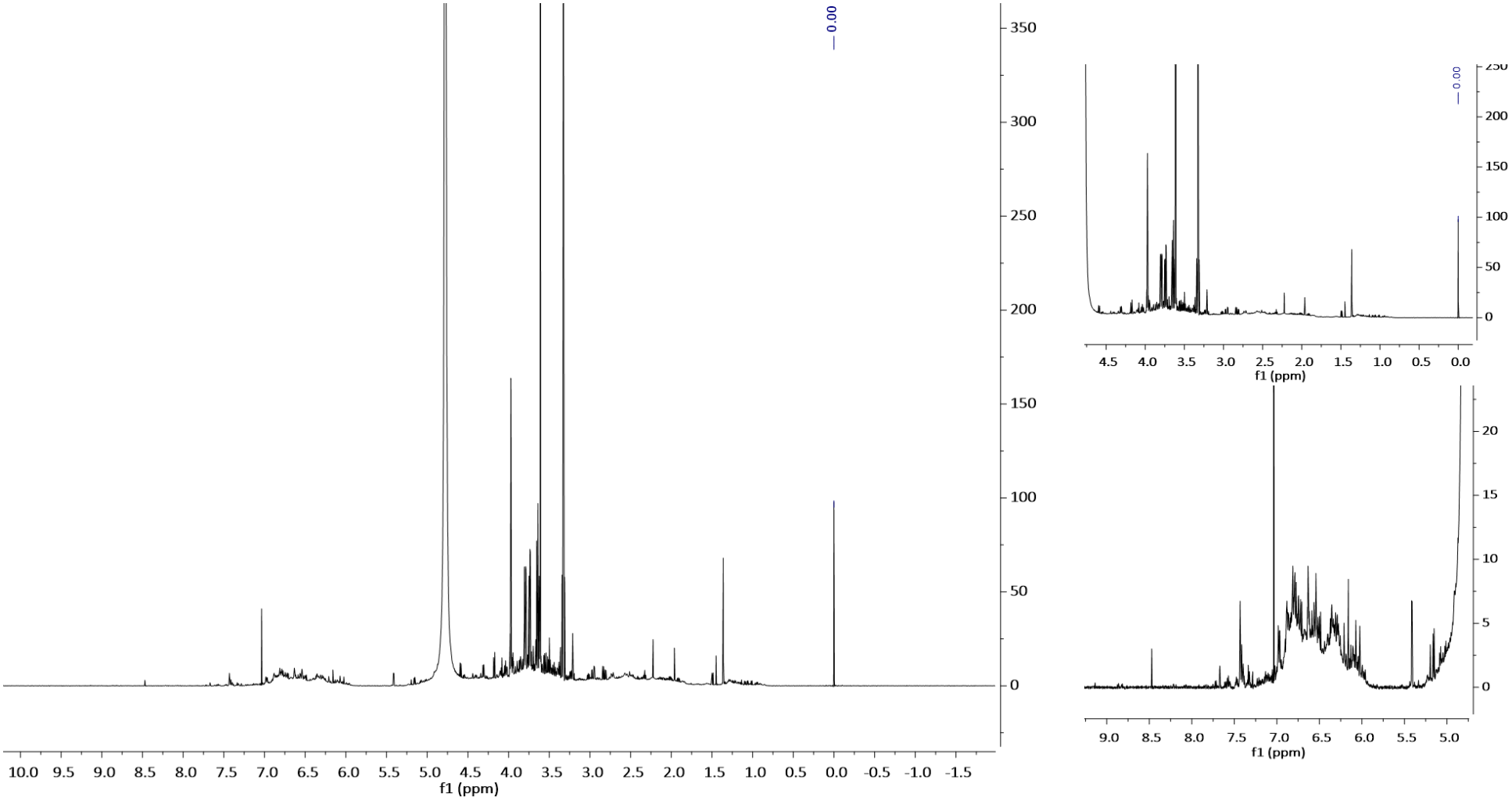


Figure 6.14 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Senna petersiana* acquired on a 600 MHz NMR.

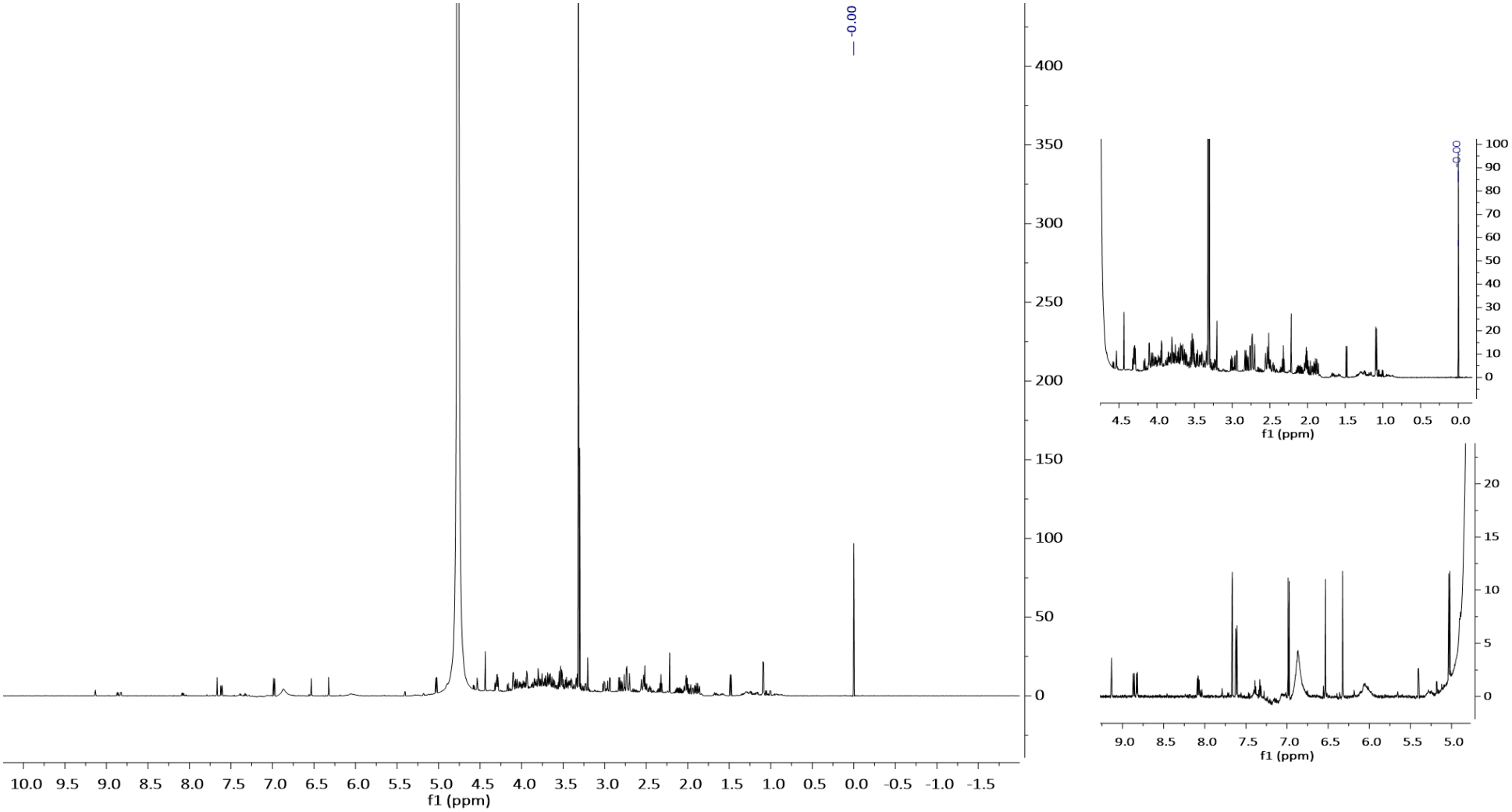


Figure 6.15 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Adansonia digitata* acquired on a 600 MHz NMR.

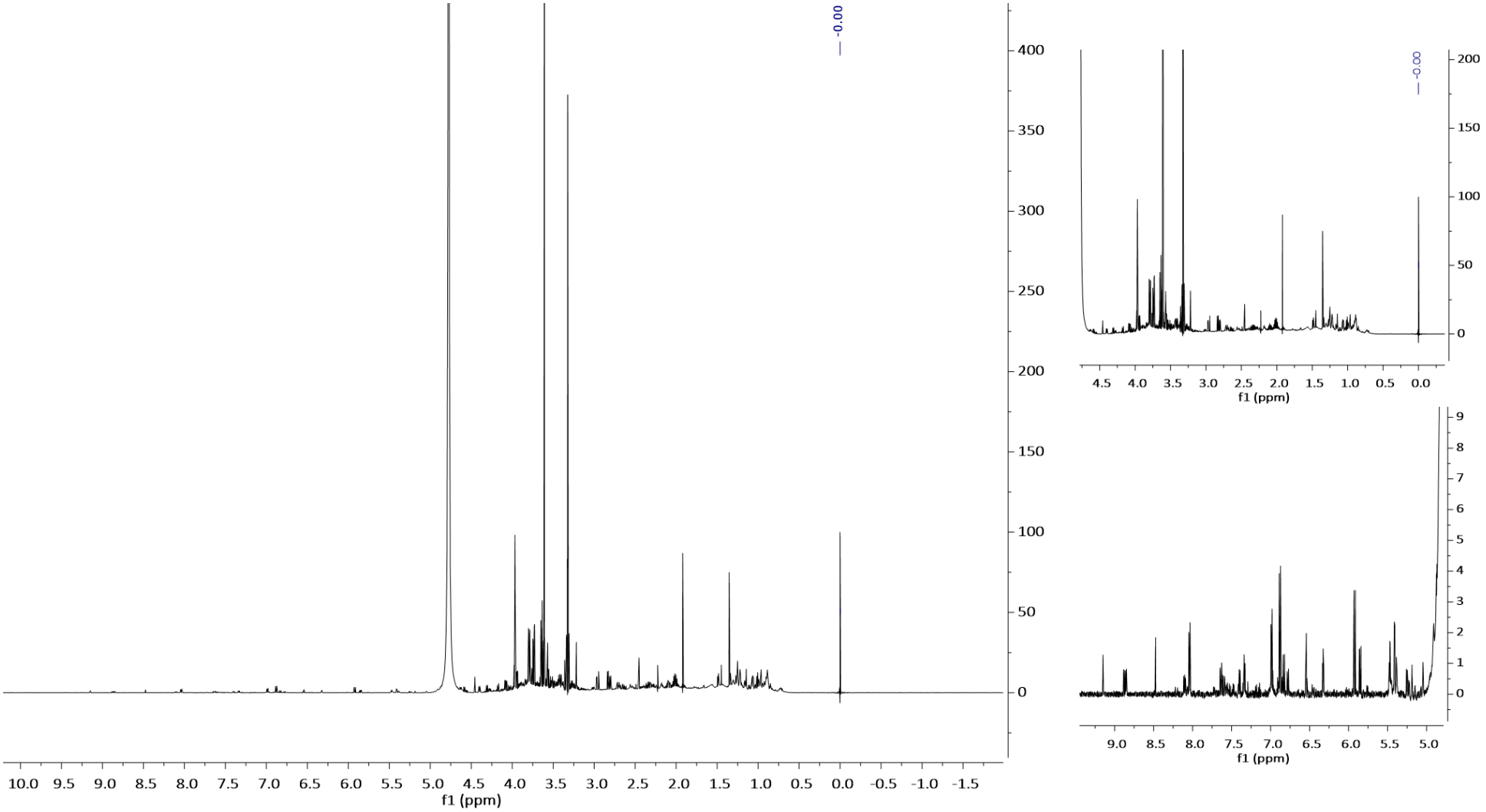


Figure 6.16 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Sutherlandia frutescens* acquired on a 600 MHz NMR.

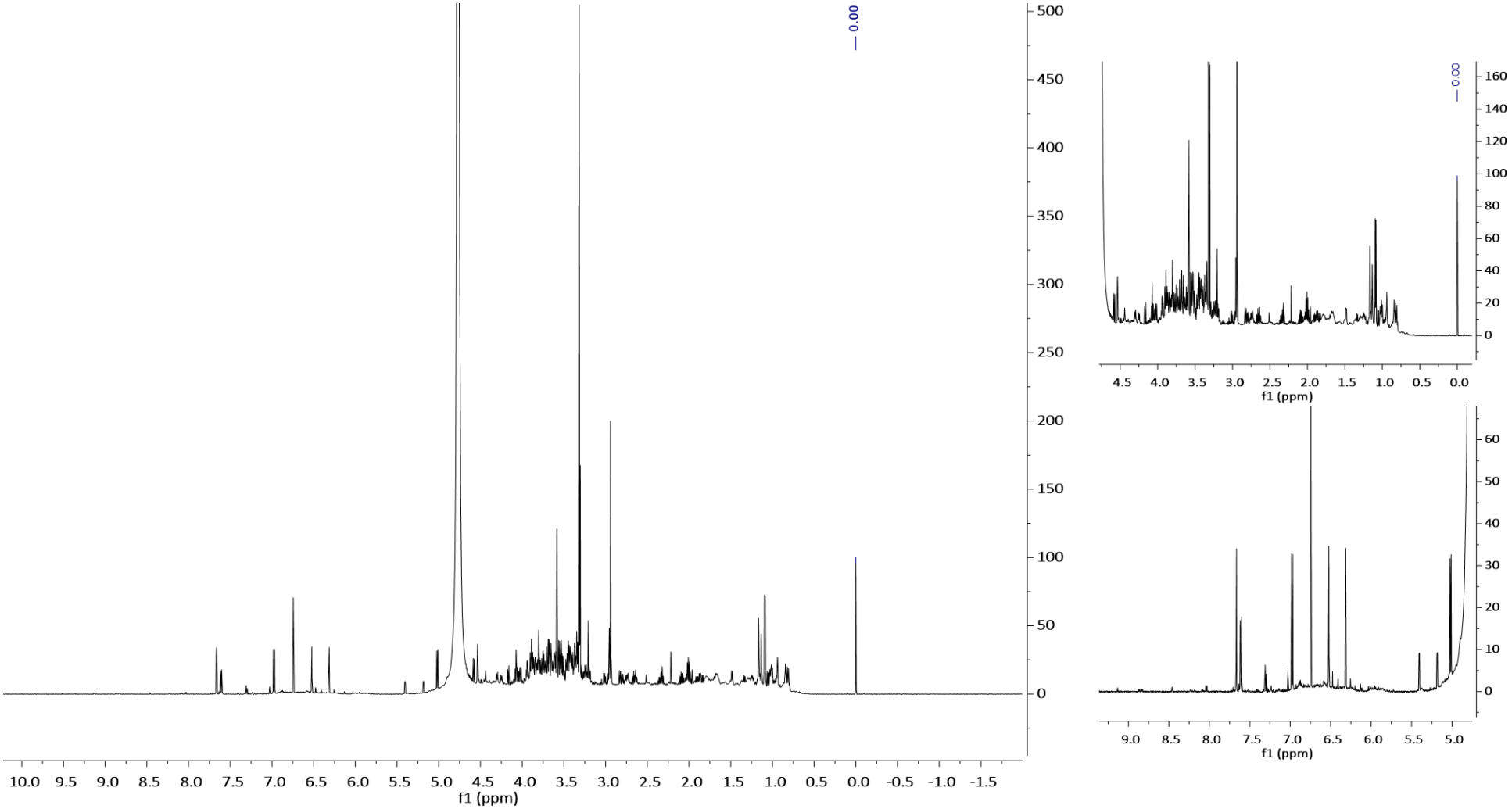


Figure 6.17 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Ziziphus mucronata* acquired on a 600 MHz NMR.

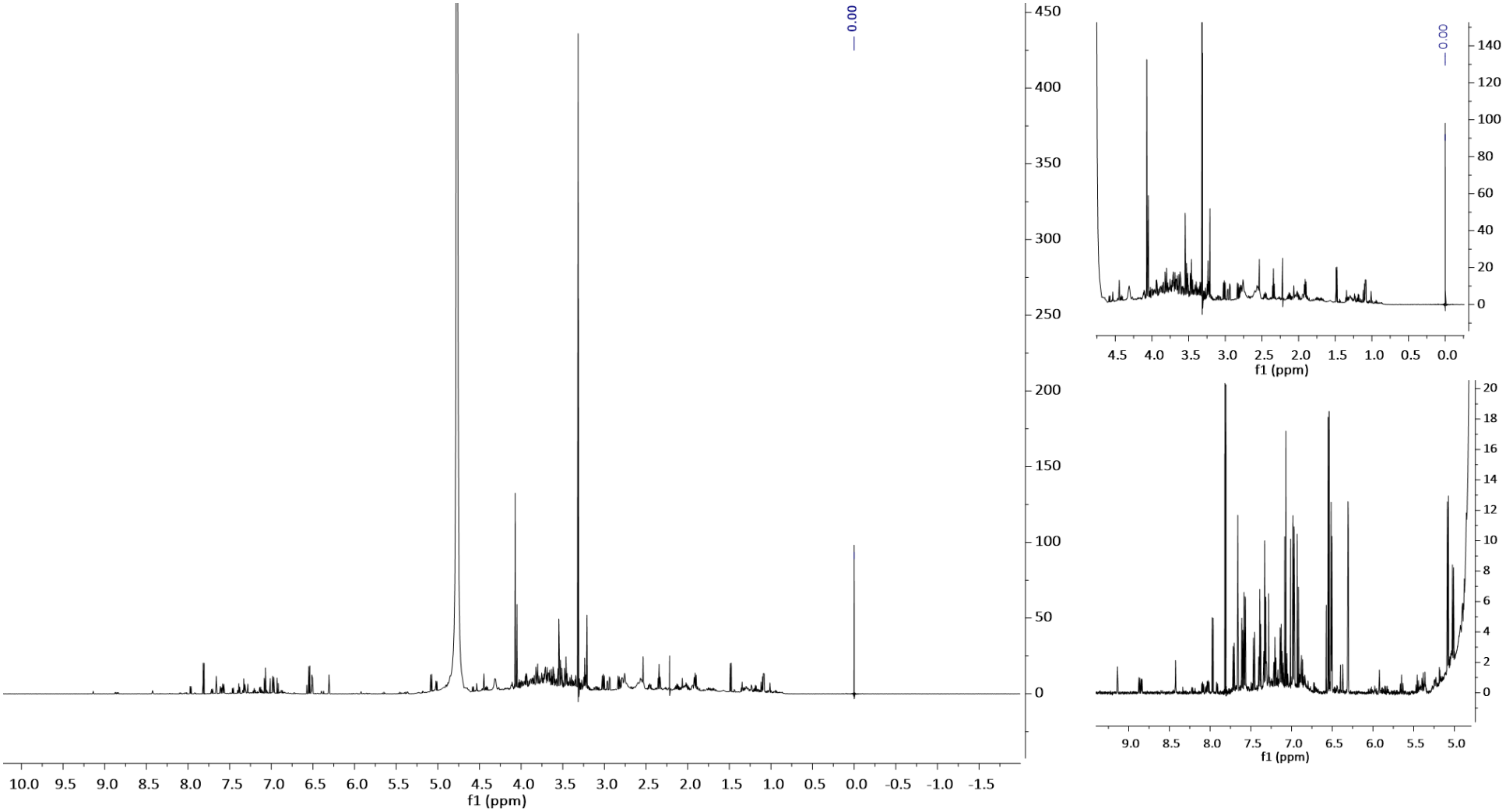


Figure 6.18 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Ricinus communis* acquired on a 060 MHz NMR.

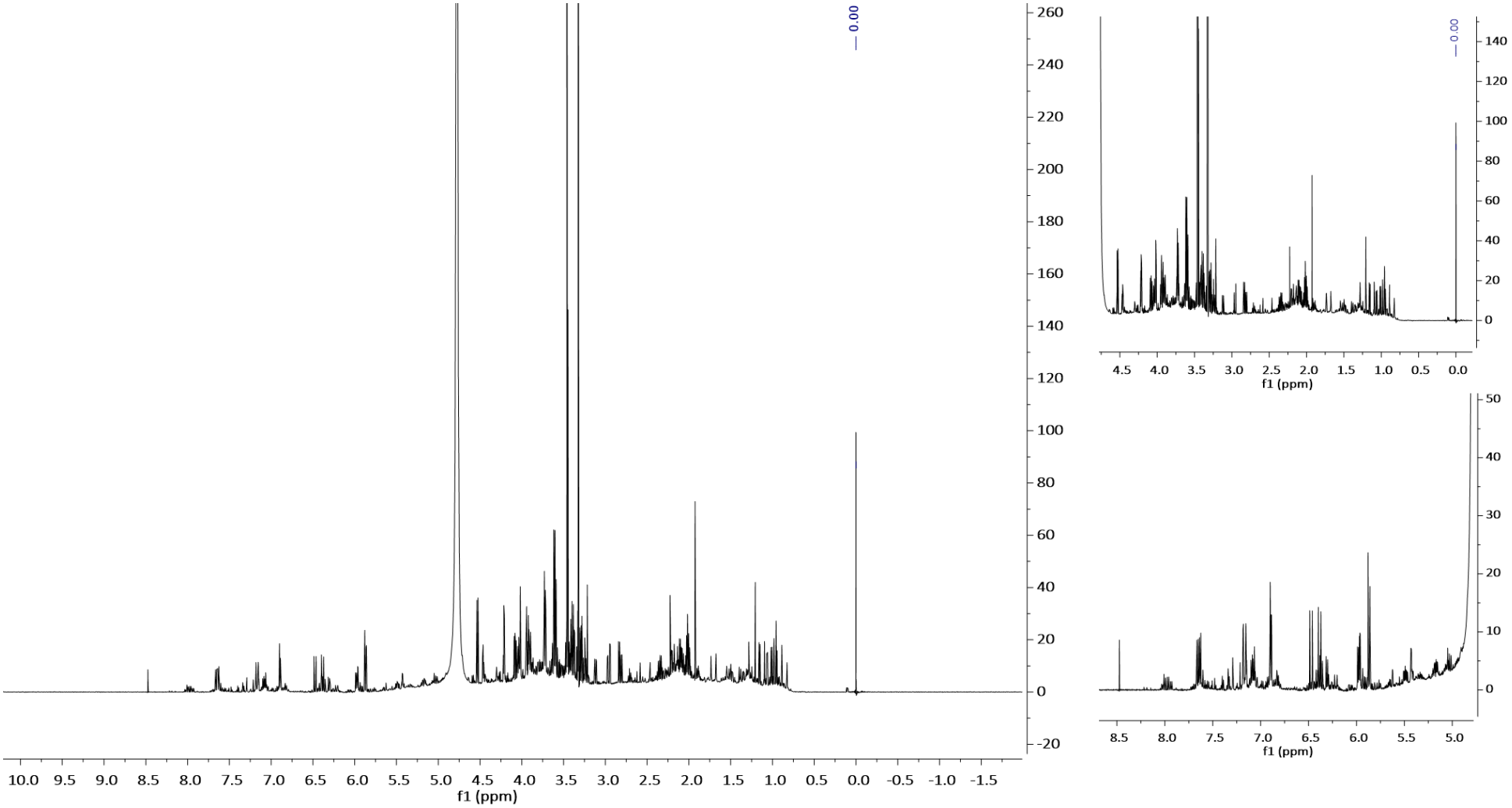


Figure 6.19 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Artemisia afra* acquired on a 600 MHz NMR.

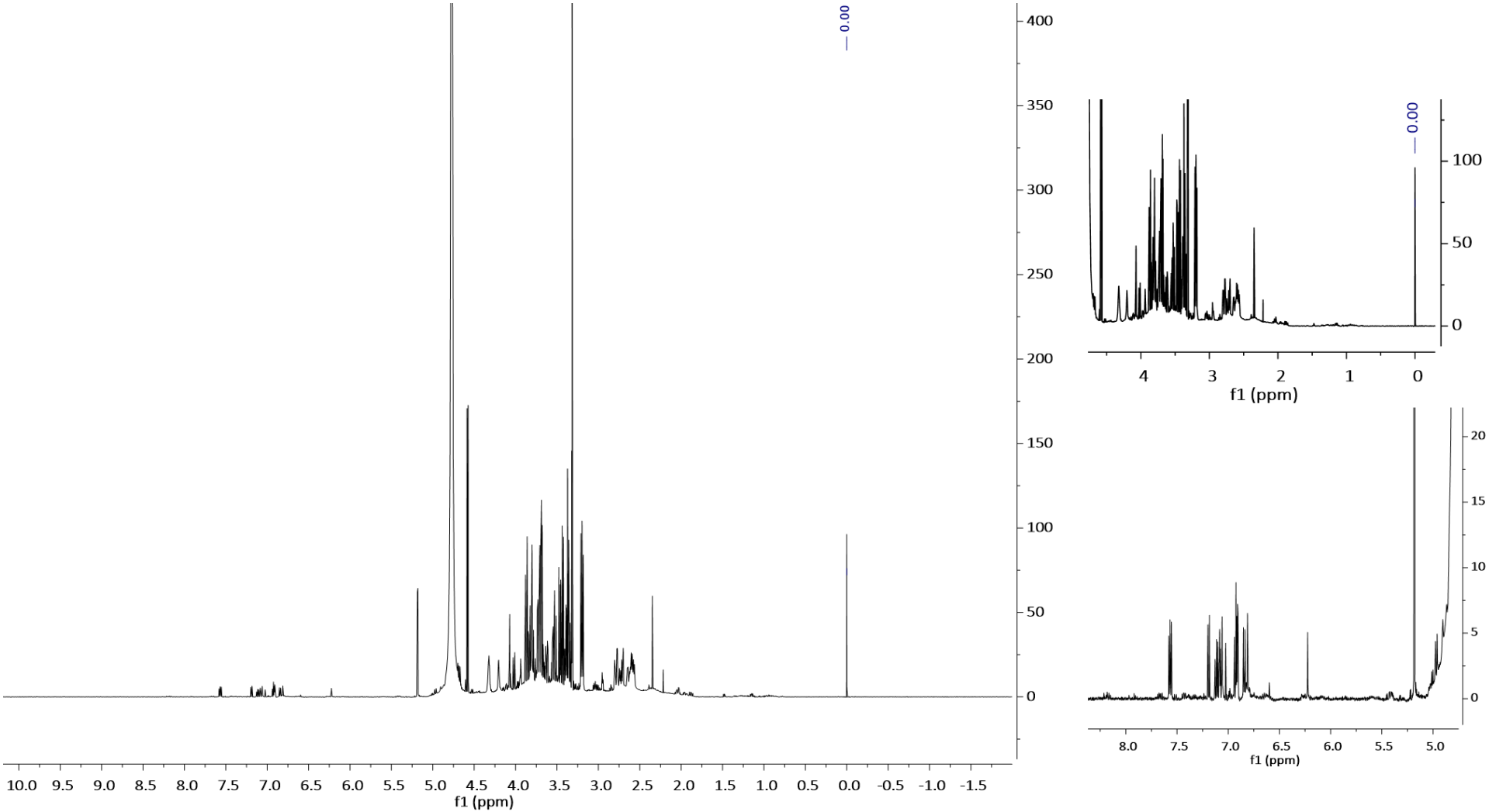


Figure 6.20 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Aloe ferox* acquired on a 600 MHz NMR.

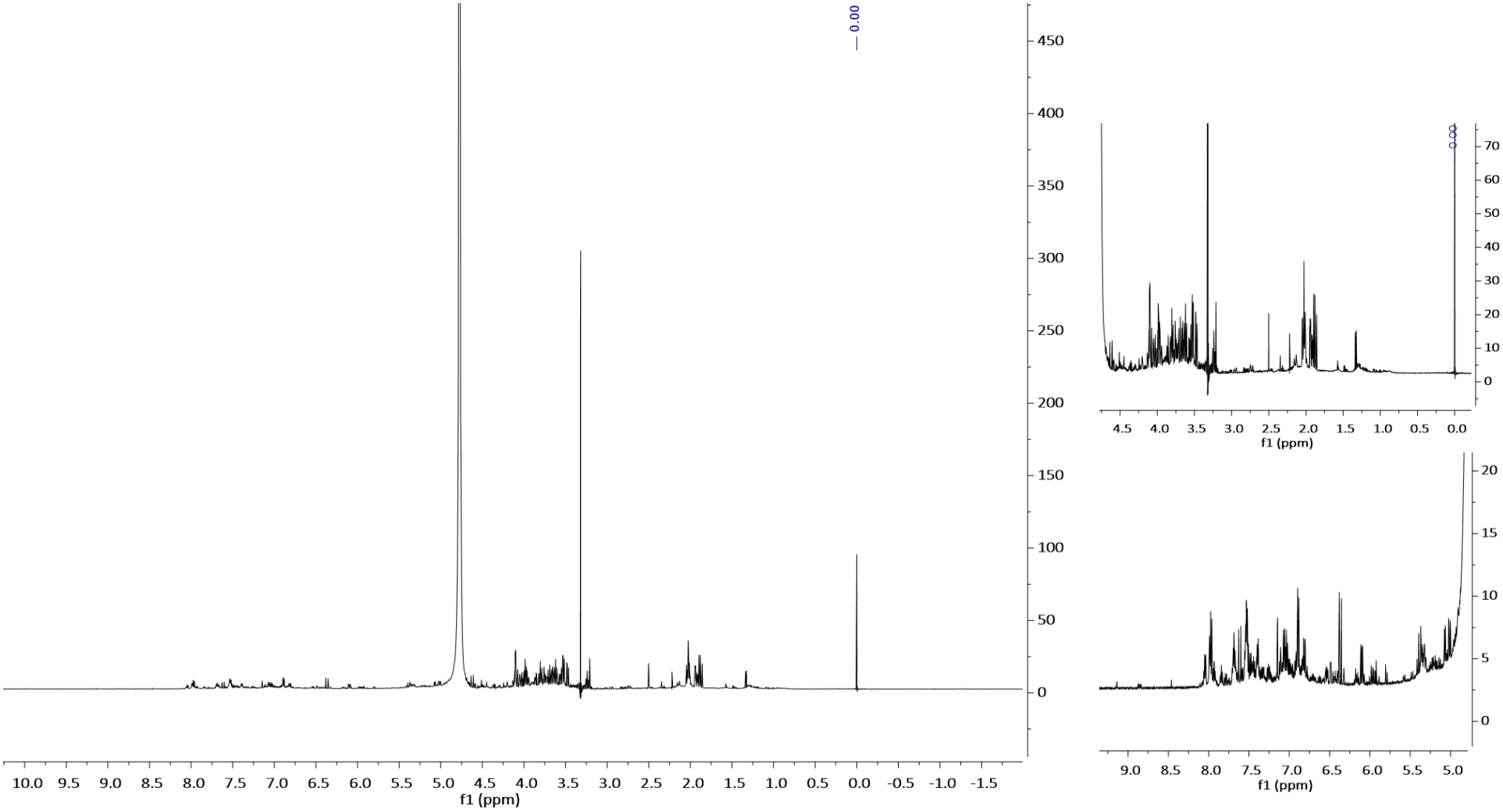


Figure 6.21 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Prunus africana* acquired on a 600 MHz NMR.

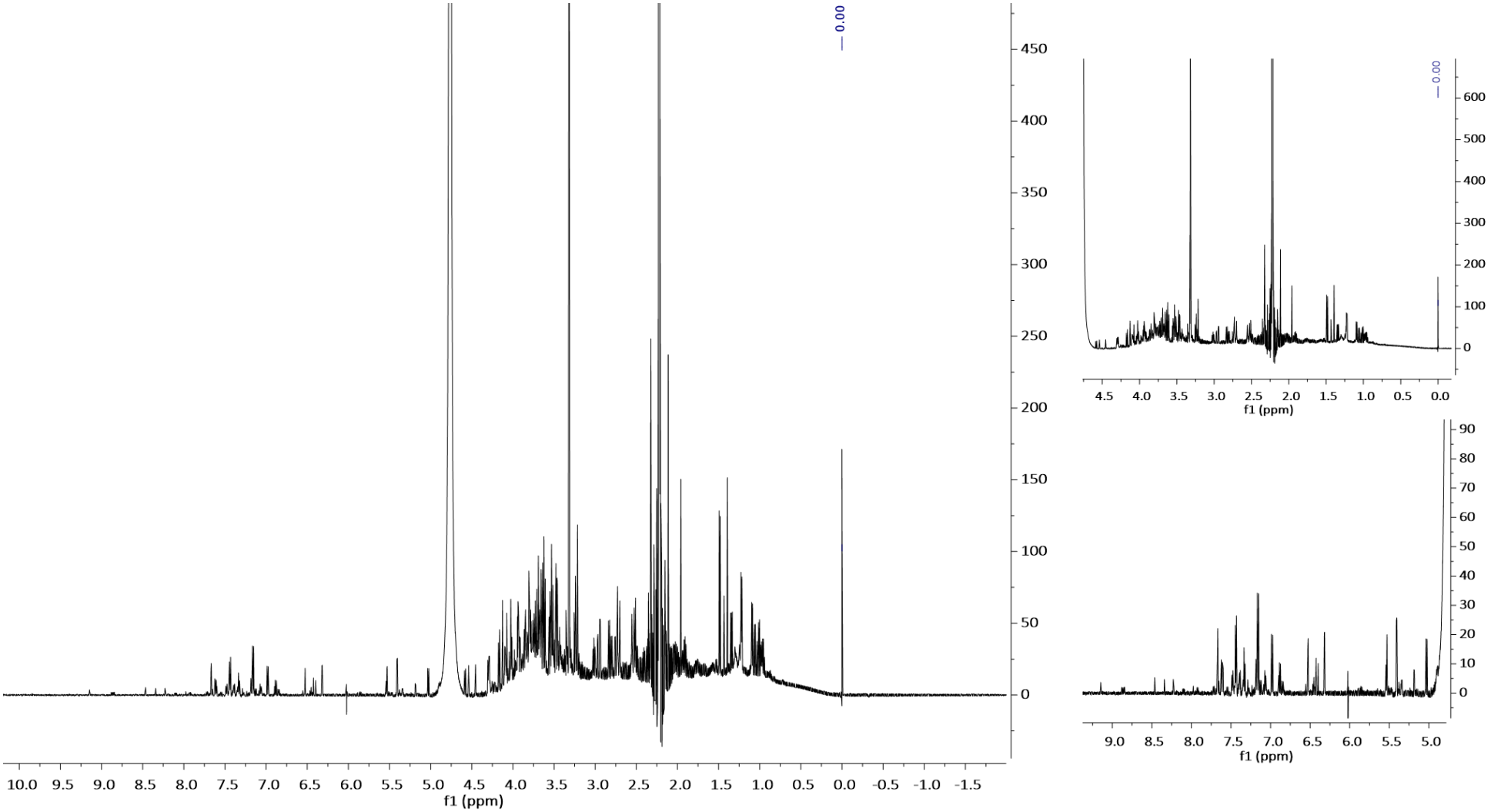


Figure 6.22 Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 50 % aqueous-methanol plant extract of *Moringa oleifera* acquired on a 600 MHz NMR.